

TRANSMITTAL LETTER TO THE UNITED STATES

DESIGNATED/ELECTED OFFICE (DO/EO/US)

CONCERNING A FILING UNDER 35 U.S.C. 371

LWEP:103_US

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

unknown 09/868129

INTERNATIONAL APPLICATION NO.

PCT/DE99/03768

INTERNATIONAL FILING DATE

27-November-1999

PRIORITY DATE CLAIMED

17-December-1998

TITLE OF INVENTION

**METHOD FOR THE INDIVIDUAL ADAPTATION OF EXCITATION INTENSITIES IN A MULTIBAND
FLUORESCENCE MICROSCOPE AND MULTIBAND FLUORESCENCE MICROSCOPE FOR CARRYING OUT
SAID METHOD**

APPLICANT(S) FOR DO/EO/US

Albrecht WEISS

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☐ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☒ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☒ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☒ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☒ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

1) Express Mail Label No. EL 917291338 US

2) Acknowledgement Postcard

3) Assignee for Purposes of Publication: Leica Microsystems Wetzlar GmbH
Wetzlar, Germany

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

INTERNATIONAL APPLICATION NO.

ATTORNEY'S DOCKET NUMBER

09/868129

PCT/DE99/03768

LWEP:103_US

24. The following fees are submitted:

CALCULATIONS PTO USE ONLY

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00
- ☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

\$860.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

\$0.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	21 - 20 =	1	x \$18.00
Independent claims	2 - 3 =	0	x \$80.00

\$18.00

\$0.00

Multiple Dependent Claims (check if applicable). ☐

\$0.00

TOTAL OF ABOVE CALCULATIONS =

\$878.00

☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.

\$0.00

SUBTOTAL =

\$878.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).

\$0.00

TOTAL NATIONAL FEE =

\$878.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable). ☒

\$40.00

TOTAL FEES ENCLOSED =

\$918.00

Amount to be:	\$
refunded	
charged	\$

- ☒ A check in the amount of \$918.00 to cover the above fees is enclosed.
- ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0822. A duplicate copy of this sheet is enclosed.
- ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

George L. Snyder, Jr.
 Registration No. 37,729
 Simpson, Simpson & Snyder, L.L.P.
 5555 Main Street
 Williamsville, New York 14221
 Telephone No. (716) 626-1564
 Facsimile No. (716) 626-0366



24041

PATENT TRADEMARK OFFICE

SIGNATURE

George L. Snyder, Jr.

NAME

37,729

REGISTRATION NUMBER

June 14, 2001

DATE

Express Mail Label No. EL 917291338 US

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
PATENT COOPERATION TREATY**

Applicant(s): Albrecht WEISS Atty Ref: LWEP:103_US_
Serial No.: unknown Group Art Unit: unknown
Filing Date: unknown Examiner: unknown
Title: METHOD FOR THE INDIVIDUAL ADAPTATION OF EXCITATION
INTENSITIES IN A MULTIBAND FLUORESCENCE MICROSCOPE AND
MULTIBAND FLUORESCENCE MICROSCOPE FOR CARRYING OUT
SAID METHOD

International Application No.: PCT/DE99/03768
International Filing Date: November 27, 1999

PRELIMINARY AMENDMENT

Box PCT
Commissioner for Patents
Washington, D.C. 20231

Honorable Sir:

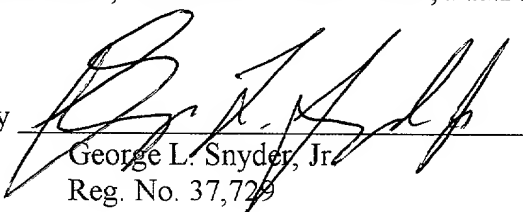
Please preliminarily amend the above-identified application concurrently filed under 35
USC 371 as follows:

IN THE SPECIFICATION:

Please enter the enclosed Substitute Specification, which is provided herewith in both a
Marked Version showing changes from the English translation of the International Application
and an Unmarked Version. The Substitute Specification contains no new matter.

Respectfully submitted,
SIMPSON, SIMPSON & SNYDER, L.L.P.

By


George L. Snyder, Jr.
Reg. No. 37,729

GLS/
Encs.

Preliminary Amendment - LWEP:103_US_
June 14, 2001
Page 2 of 2

5555 Main Street
Williamsville, New York 14221
Telephone: (716) 626-1564
Facsimile: (716) 626-0366

Dated: June 14, 2001

TITLE OF THE INVENTION

Method for the Individual Adaptation of Excitation Intensities in a Multiband
Fluorescence Microscope and a Multiband Fluorescence Microscope for Carrying Out
Said Method

5

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application is the U.S. national phase under 35 U.S.C. 371 of
International Application No. PCT/DE99/03768 filed November 27, 1999 claiming
priority of German Patent Application No. 198 58 206.4 filed December 17, 1998.

10

FIELD OF THE INVENTION

[0002] The invention relates to a method for the individual adaptation of excitation
intensities in a multiband fluorescence microscope and a multiband fluorescence
microscope to execute the method corresponding to the features of the introductory clause
of the independent claims.

15

BACKGROUND OF THE INVENTION

[0003] With the multiband fluorescence microscope, the user frequently confronts the
problem that the different fluorescence bands in the microscopic image have varying
intensities and are not uniformly visible. The cause lies frequently in the difference in
excitation intensities in the illumination beam path or even in the varying blockage of the
fluorescence intensities by a barrier filter in the imaging beam path. Also, different
concentrations of the fluorescence die for the different excitation bands, even when
staining the objects to be considered, or the progressive bleaching of the dye, the so-
called fading, lead to differing intensities of the fluorescence bands in the microscopic
image. The different intensities of the fluorescence bands prove to be especially
problematic then if the microscopic image is to be photographically recorded. Then the

20

25

portion of the fluorescent light of weak intensity on the photo is too weakly reproduced or is not visible at all. Only with intensities of the fluorescence bands that are as uniform as possible can there be defect-free photos of the microscopic image.

5 [0004] US Patent 5,371,624 specifies a fluorescence microscope having only two excitation bands in which the intensities of the two excitation bands can be intermittently affected. It includes an illumination beam path having a light source and an excitation filter that produces several excitation bands of varying light wavelengths from the light of the light source. Furthermore, it has a splitter mirror, an output filter (also designated as a barrier filter or emission filter) for the fluorescent light and a filter element for affecting
10 the intensities of the excitation bands.

[0005] The filter element can, by tilting continuously with respect to the optical axis, be switched between two limit positions having two fixed, predetermined values of the transmission factors of one or the other excitation band. In the one limit position, only the first excitation band is attenuated, in the other limit position only the second
15 excitation band is attenuated and between the two limit positions, neither of the excitation bands is attenuated. A drop in the transmission factor of the particular excitation band is only possible up to the fixed, predetermined value. However, variation between a maximum transmission and a zero value, i.e., up to full suppression of one of the two excitation bands, is not possible. Moreover, only two excitation bands can be affected.

20

SUMMARY OF THE INVENTION

[0006] It is object of the present invention to specify a method for individual adaptation of excitation intensities in a multiband fluorescence microscope and a multiband fluorescence microscope to execute the method, in which one or more of the
25 excitation bands can be partially or completely filtered out. For this purpose the transmission factor for each excitation band is to be continuously adjustable using simple means.

[0007] This objective is met by the invention via the characterizing features of the independent claims. Advantageous embodiments arise from the features of the dependent claims.

5 [0008] The method of the invention starts from a known multiband fluorescence microscope in which several excitation bands of varying wavelengths are produced using an excitation filter in an illumination beam path from the light of a light source. The excitation bands illuminate a fluorescence object prepared using fluorescence dyes and are converted by it into frequency-shifted fluorescence bands.

10 [0009] According to the invention, the fluorescence intensities of the individual fluorescence bands are first determined in the microscopic image and compared to previously set intensity setpoint values. The fluorescence intensities can be determined, for example, either visually or by using an intensity meter. This can consist for example of a video or CCD camera having an image analysis system connected in an outgoing circuit.

15 [0010] In this context a different intensity setpoint value can be set for each fluorescence band. However, in practice, the setpoint values are oriented toward concrete problems posed by the microscope user. If, for example, the microscopic image is to be documented either photographically or by video camera, and thus each fluorescence band in the photo or in the video image is to be reproduced with equal brightness, then the
20 level of the setpoint values depends on the spectral sensitivity of the film or the camera. Therefore, their spectral sensitivity must be taken into consideration in the determination of setpoint values for the various excitation bands.

[0011] Therefore, the desired setpoint values must all be equal – and in particular equal to the lowest fluorescence intensity – provided that the film or the camera
25 reproduces all spectral colors with equal intensity. However, if the spectral sensitivity of the video camera or of the film is not constant, then correspondingly varying setpoint values must be set for the different fluorescence bands in order to be able to reproduce the fluorescence bands with equal brightness.

[0012] On the other hand, if specific fluorescence bands do not appear on the photo or the video image, and thus are masked out, then the setpoint values for said bands must be equal to zero. In this context, it proves to be beneficial if the setpoint values are equal to the lowest of their intensities also for the fluorescence bands that are not masked out.

5 Then these fluorescence bands all appear equally bright.

[0013] For each excitation band that is assigned to a fluorescence intensity deviating from the setpoint values, a selective filter according to the present invention tuned to the pertinent excitation band is brought into the illumination beam path. Its spectral transmission curve is configured so that exclusively the intensity of the pertinent
10 excitation band is reduced, but the remaining spectral regions pass through unhindered.

[0014] In a multiband fluorescence microscope according to the invention for executing the method according to the invention, a filter draw assembly made of a multiplicity of individually movable filter draws is perpendicularly inserted in the illumination beam path tightly next to the aperture diaphragm plane.

15 **[0015]** The structure of the filter draw is a function of the number of the different excitation bands of the multiband fluorescence microscope. For a number of n excitation bands, each filter draw has n selective filters harmonized to the excitation bands and having surface regions with high and low transmission factors.

[0016] The different transmission factors are achieved by virtue of the fact that only
20 certain area portions of the beam cross-section are occupied using separate filter-area elements. In this context as even a surface coverage of the beam cross-section as possible is sought so that no unilateral shading, and thus also no unilateral illumination of the pupils, is made. As a result a crooked illumination, and thus a lateral migration of the picture elements, is prevented during focusing.

25 **[0017]** It must be possible to insert the filters in the illumination beam path independently of each other individually or in combination using the desired surface region or the desired transmission. In this context, it must be possible with n excitation bands to combine a maximum of $n-1$ filters with each other, i.e. simultaneously insert

them in the illumination beam path. This is sufficient since all excitation bands must never be attenuated simultaneously, because as a rule an excitation band supplies the intensity setpoint value and remains unchanged. Likewise, all excitation bands must not be triggered simultaneously, since this is equivalent to turning off the lighting.

5 [0018] In order to achieve the required combinations from $n-1$ filters, $n-1$ individually movable filter draws that are situated tightly parallel next to the aperture diaphragm plane with little distance between them and are effectual in combination are arranged in an advantageous embodiment of a multiband fluorescence microscope according to the invention having n excitation bands on $n-1$ disk planes. With each filter draw, one filter
10 for an excitation band can be suitably adjusted so that altogether a maximum of $n-1$ excitation bands (one less than the maximum number) can be selectively attenuated or triggered.

[0019] For two excitation bands, it is sufficient if the two required filters are arranged in a single disk plane ($n-1=1$), since either only the one or only the other filter must be
15 inserted in the illumination beam path. Therefore, a filter draw assembly to affect two excitation bands is preferably of one piece, thus designed with only one filter draw. However, for more than two excitation bands, a corresponding multi-piece filter draw assembly must be provided with $n-1$ individually movable filter draws.

[0020] Except for the selective filters for the various excitation bands, each filter
20 draw has at least one blank aperture with the beam diameter of the illumination beam path. In this case a blank aperture is arranged next to each filter. Depending on the embodiment, the filters can also be grouped directly around a single blank aperture. The transmission factor of the filter diminishes in the shifting direction as the distance from the blank aperture increases. In this case each filter has a surface region with the lowest
25 transmission factor and a minimum diameter equal to the beam diameter x . If this surface region is inserted in the illumination beam path, the associated excitation band is maximally attenuated, i.e., to the zero value.

[0021] By correctly positioning each of the individual filters in the illumination beam path, the transmission factor that is acting on the illumination beam path is precisely individually adjusted such that by attenuating the excitation band assigned to the filter, the resulting fluorescence intensity matches its intensity setpoint value. To execute this process step, separate positioning means, which bring about the full or partial covering of the illumination beam path using the appropriate surface region of the filter draw, are assigned to each filter draw. Thus, the blank aperture or one or more of the filters or combinations of filter regions and the blank aperture can be inserted in the illumination beam path, according to preference. If this last process step was executed for all filters and thus all excitation bands, all fluorescence intensities match their setpoint values.

[0022] Even after optimal setting of the fluorescence intensities by applying the method according to the invention using a specified multiband fluorescence microscope, deviations of the fluorescence intensities from the setpoint values reoccur after some time. This is attributable to the fact that the various fluorescence dyes for the various excitation bands fade at differing rates, i.e., they exhibit a specific fading.

[0023] Therefore, in one advantageous embodiment of the method, the time changes of the fluorescence intensities are continuously determined. The transmission factors of the filter that are in effect within the illumination beam path are then repeatedly adjusted at specific time intervals so that the fluorescence intensities are always brought into accord with their setpoint values.

[0024] An especially advantageous embodiment of the method provides for an automatic fading compensation. For this purpose the time modifications of the fluorescence intensities are automatically continually determined. To do this the microscopic image, for example, can be automatically continually recorded using a video camera and the fluorescence intensities contained therein can be determined using an image analysis system and compared to the predetermined setpoint values.

[0025] Then the transmission factors of the filter are automatically continuously modified and adapted so that the fluorescence intensities are always kept at the intensity

setpoint values. To this end, motorized positioning means, for example, can be used. The drive of the motors and regulation of the fluorescence intensities in relation to the setpoint values can be achieved using an electronic unit and a computer to which the signals of the video camera or the image analysis system are fed.

5

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] The invention is explained in more detail below with respect to the schematic drawing. Shown are:

- 10 Figure 1 a beam path of a multiband fluorescence microscope according to the invention;
- Figure 2a-d various embodiments of a filter draw for a dual-band fluorescence microscope;
- Figure 2e the spectral transmission curve of the excitation bands and the assigned filter of a filter draw for a dual-band fluorescence microscope;
- 15 Figure 3a-c various embodiments of a filter draw for a two-piece filter draw assembly of a three-band fluorescence microscope;
- Figure 3d the spectral transmission curve of the excitation bands and the assigned filter of a filter draw for a three-band fluorescence microscope;
- Figure 4a-b various embodiments of a filter draw for a three-part filter draw assembly of a four-band fluorescence microscope;
- 20 Figure 4c the spectral transmission curve of the excitation bands and the assigned filter of a filter draw for a four-band fluorescence microscope.

DETAILED DESCRIPTION OF THE INVENTION

- 25 [0027] Figure 1 shows a beam path of a multiband fluorescence microscope for executing the method according to the invention. From a light source 1 an illumination beam path starts with an optical axis 2 in which a collector 3, a first lens member 4, an aperture diaphragm 5, a second lens member 6, a radiant field stop 7, a third lens member

8 and a excitation filter 9 are arranged in succession to produce the excitation bands. The illumination beam path is deflected at a beam splitter 10 to an objective 11. It passes through the objective 11 and reaches a fluorescence object 12, which rests on a specimen stage 13.

5 **[0028]** The excitation bands produced by excitation filter 9 in the illumination beam path are converted into fluorescence bands 12 by fluorescence dyes that were injected in fluorescence object 12, and said bands are emitted by the fluorescence object in a frequency-shifted state. This fluorescence light passes through objective 11, beam splitter 10, an output filter 14, a tube lens 15 and reaches an intermediate image plane 16.

10 The intermediate image produced here can be considered by microscope user using an eyepiece 17. The intermediate image is displayed via a TV output on a video camera 18 having an image analysis system 19 connected in series. With this design the fluorescence intensities of the individual fluorescence bands in the microscopic image can be determined either visually using eyepiece 17 or using image analysis system 19 and then compared to intensity setpoint values greater than or equal to zero.

15 **[0029]** To attenuate the excitation bands, a filter draw assembly 20 according to the invention, consisting in this example of two filter draws 21a and 21b, is inserted perpendicular to the optical axis 2 of the illumination beam path tightly next to, i.e., behind or in front of aperture diaphragm plane 24. Arranged on each filter draw 21a, 21b are a blank aperture 22 and in addition several selective filters 23 tuned to the excitation bands. By arranging filter draws 21a, 21b as narrowly as possible, filters 23 can be situated as tightly as possible next to aperture diaphragm plane 24. This ensures that the inserted filter draws 21a, 21b are not visible in the image. A further advantage is that in this case the least possible beam fanning occurs, so that the surface region of filter 23 can be kept as small as possible.

25 **[0030]** According to the present invention, positioning means are assigned to each filter draw. In this context positioning means having the possibility of manual adjustability can be used in economic embodiments. Although motorized positioning

means are more expensive, they permit an automation of the method according to the invention.

[0031] In the embodiment illustrated here, two motors 25a, 25b that can shift parallel to the aperture diaphragm plane and/or rotate filter draws 21a, 21b are arranged as positioning means for filter draws 21a, 21b. Using the motors 25a, 25b either the blank aperture 22 or a selective filter 23 or a combination of the two can be inserted in the illumination beam path by the particular assigned filter draw 21a or 21b. The motors 25a, 25b are driven by control electronics 26, which receive the input signal from the image analysis system 19.

[0032] The image analysis system 19 determines from the camera signal the deviation of the fluorescence intensities from their setpoint values. Then those excitation bands that cause the deviations from setpoint values are determined and one of the filter draws 21a, 21b is assigned to each of these excitation bands for attenuation. In the illustrated example, two excitation bands can thus be completely attenuated, i.e. to the zero value, or even partially attenuated. For each filter draw 21a, 21b a drive signal is produced for the associated motor 25a, 25b. Each filter draw 21a, 21b is continuously shifted via the associated motor 25a, 25b so that a filter 23 selectively acting on the assigned excitation band is inserted in the illumination beam path. Then the filter draw 21a or 21b is shifted further until a surface region of the inserted filter 23 has a transmission factor in the illumination beam path through which the assigned excitation band is attenuated to the intensity setpoint value. In this way, all excitation bands are set to their intensity setpoint values.

[0033] Different embodiments of filter draw assembly 20 or the filter draw 21 and the associated spectral transmission curves of the filters arranged on them are explained below.

[0034] A filter draw 21 for a multiband fluorescence microscope having two excitation bands A and D is depicted in Figure 2a. Applied to a rectangular glass plate 27 as different vapor deposition layers are a long-pass filter 29 for the intensity reduction of

the short-wave excitation band A and a short-pass filter 28 for the intensity reduction of the long-wave excitation band D. They are located on the ends of the glass plate 27, and between them is the blank aperture 22 in the center of filter draw 21. Its cross-section is equal to the beam diameter x in the aperture diaphragm 5 of the illumination beam path.

- 5 Filter draw 21 can be continuously shifted by positioning means (not shown) longitudinally in both directions parallel to the aperture diaphragm plane 24. The movement is indicated by a double arrow.

[0035] Short-pass filter 28 and long-pass filter 29 are each applied in this version as a connected vapor-deposition layer. The transmission of the two filters 28, 29 is largest right next to blank aperture 22 and gets smaller as the distance from blank aperture 22 increases. To this end next to blank aperture 22 filter draw 21 is not completely covered with filters 28, 29, but rather the portion of the filter area increases as one moves from blank aperture 22 toward the end of filter draw 21.

[0036] Thus, the vapor-deposition layers on the ends of filter draw 21 form a rectangle with a minimum edge length equal to the beam diameter x . If this vapor-deposited rectangular area completely covers the illumination beam path, the smallest percentile is thus set and the accompanying excitation band is completely attenuated, i.e., to the zero value. The vapor-deposited rectangular area borders the base of vapor-deposited triangular area, whose opposing corner faces blank aperture 22.

20 [0037] Via the vapor-deposited triangular areas, which always cover the beam diameter only partially, the transmission factor of short-pass filter 28 and long-pass filter 29 in each case diminishes as one moves from blank aperture 22 toward the ends of filter draw 21. By inserting any fractional area of short-pass filter 28 or of long-pass filter 29, any desired transmission factor of the filter 28, 29 in the illumination beam path can be realized and thus one or the other excitation band can be weakened or faded out according to preference.

[0038] A filter draw 21 for a multiband fluorescence microscope according to the invention having two excitation bands A and D is likewise depicted in Figure 2b.

Applied to a rectangular glass plate 27 are a short-pass filter 28 for filtering the long-wave excitation band A and a long-pass filter 29 for filtering the short-wave excitation band D as different vapor-deposition layers.

5 [0039] Here, as in Figure 2a, the vapor-deposition layers in the direction of blank aperture 22 have a triangular contour. However, the triangles in this case are each not vapor-deposited as connected areas, but rather as small area elements 30 of equal size. In this way a longer adjustment range with higher transmissions is achieved for the two filters 28, 29. At the ends of filter draw 21, the vapor-deposition layers form a rectangle having a minimum edge length equal to the beam diameter x and thus a region of the least
10 transmission with which the assigned excitation band can be attenuated to zero.

[0040] Another filter draw 21 for a multiband fluorescence microscope according to the invention having two excitation bands A and D is depicted in Figure 2c. Applied to a rectangular glass plate 27 are a short-pass filter 28 for filtering the long-wave excitation band A and a long-pass filter 29 for filtering the short-wave excitation band D as different
15 vapor-deposition layers. Located between them is a blank aperture 22.

[0041] The filters 28, 29 are vapor-deposited in this version of filter draw 21 next to blank aperture 22 as separate area elements 30 formed in any way desired (here circular).

[0042] In this context, the size of area elements 30 and thus the portion of the area of filter draw 21 increases when moving from blank aperture 22 to the ends of filter draw
20 21. The transmission factor of filters 28, 29 is largest next to blank aperture 22 and diminishes when moving in the direction toward the ends of filter draw 21. On both ends of filter draw 21, each of the two filters 28, 29 have a completely vapor-deposited area with the smallest transmission factor, said area having at least the diameter x of the beam path.

25 [0043] Another filter draw 21 for a multiband fluorescence microscope according to the invention having two excitation bands A and D is depicted in Figure 2d. Applied to a rectangular glass plate 27 are a short-pass filter 28 for filtering the long-wave excitation

band A and a long-pass filter 29 for filtering the short-wave excitation band D as different vapor-deposition layers. Located between them is a blank aperture 22.

[0044] In this embodiment the vapor-deposition layers are applied as strip-shaped area elements 30, the width of the strips increasing as one moves from the center to the ends of filter draw 21. In this way the transmission diminishes as one moves from the blank aperture 22 toward the ends of filter draw 21. Also here the two filters 28, 29 each have a completely vapor-deposited surface with the lowest possible transmission factor at both ends of filter draw 21, said surface having at least the diameter x of the beam path.

[0045] The spectral transmission curves of the short-wave excitation band A and the long-wave excitation band D, as well as the transmission curves T_A , T_D of the associated selective filter, thus the long-pass filter for the short-wave excitation band A and the short-pass filter for the long-wave excitation band D, are depicted in Figure 2e as a function of the light wavelength λ . Filters A and B are selected so that they only selectively filter out the associated excitation band for each but allow the remaining wavelength ranges to pass through unhindered.

[0046] Figure 3a shows a rectangular filter draw 21 of a two-piece filter draw assembly 20 for a multiband fluorescence microscope having three excitation bands A, B, D. In this embodiment filter draw assembly 20 consists of two rectangular filter draws 21 identical to said bands that are tightly inserted in the illumination beam path one behind the other next to the aperture diaphragm plane 24.

[0047] Provided in the center of filter draw 21 is the blank aperture 22 having diameter x of illumination beam path 2. Applied as vapor-deposition layers on the two ends of filter draw 21 are two different combinations of two or three selective filters 28, 29, 31 for excitation bands A, B, D. The two filters 28, 31 or 29, 31 on one end are in each case arranged next to one another so that both filters border each other and blank aperture 22. In this context each filter 28, 29, 31 has an area of least transmission having at least the diameter x of illumination beam path 2. As a result filter draw 21 has at least a width of $2x$ on its short end.

[0048] The transmission factor of filters 28, 29, 31 diminishes as one moves in the longitudinal direction of filter draw 21, thus in the direction of shift from blank aperture 22 toward the ends. This is realized in the example represented here in that the vapor-deposited surface of each filter 28, 29, 31 increases as one moves from blank aperture 22 toward the end of filter draw 21.

[0049] Separate positioning means 25 for shifting filter draw 21 parallel to aperture diaphragm plane 24 in both the longitudinal and transverse directions are provided for each of the two identical shifters 21 of the filter draw assembly. As a result it is possible to insert filters 28, 29, 31, individually or in combination, completely or with only a portion of their surface in the illumination beam path.

[0050] Figure 3b shows a circular filter draw 21 of a two-piece filter draw assembly 20 for a multiband fluorescence microscope according to the invention having three excitation bands A, B, D. In this advantageous embodiment of the multiband fluorescence microscope according to the invention, filter draw assembly 20 consists of two circular filter draws 21 identical to said bands that are tightly inserted in the illumination beam path one behind the other next to the aperture diaphragm plane 24. Provided in the center of each filter draw 21 is blank aperture 22. Three selective filters 28, 29, 31 for excitation bands A, B, D are arranged bordering said opening as vapor-deposited ring sectors that border each other.

[0051] Filters 28, 29, 31 each possess a transmission factor that decreases as radius increases and is largest next to blank aperture 22. This is accomplished, for example, by virtue of filters 28, 29, 31 not being fully vapor-deposited. Instead, vapor-deposited area elements 30 that get wider as they extend radially and that have non-vapor-deposited surface regions are applied in between them next to blank aperture 22 so that the portion of vapor-deposited surface of each filter 28, 29, 31 increases as the radius increases.

[0052] Using separate positioning means (not shown here), each of the two identical filter draws 21 of filter draw assembly 20 can be shifted individually and parallel to aperture diaphragm plane 24. In this case it can either be laterally shifted in one direction

and also rotated (as indicated in Figure 3b) or, alternatively, shifted within a plane in two directions. As a result any desired fractional area of filter draw 21, for example blank aperture 22 or one of the filters 28, 29, 31 having the desired transmission factor, can be inserted in the illumination beam path. By arranging two equal filter draws 21 in the illumination beam path, any two of the three filters 28, 29, 31 can be inserted simultaneously in the illumination beam path so that two of the three excitation bands can be simultaneously weakened to the predetermined setpoint values or completely faded out.

[0053] Figure 3c shows an especially advantageous embodiment of a filter draw 21 for a two-piece filter draw assembly 20 of a multiband fluorescence microscope according to the invention having three excitation bands A, B, D. In this embodiment of the multiband fluorescence microscope, filter draw assembly 20 consists of two circular filter draws 21 identical to said bands that are inserted in the illumination beam path tightly in succession next to aperture diaphragm plane 24.

[0054] The filters 28, 29, 31 for the excitation bands A, B, D are advantageously arranged on the filter draw 21 such that it only has to be turned but not shifted (rotary movement is indicated). This simplifies the structure of the mechanical or even motorized positioning means, which are separately assigned to each of the two filter draws 21 (not shown).

[0055] In order to accomplish this, each filter draw 21 is designed with a circular shape, divided into six sectors and arranged pivoted around its center. The center of the circle lies outside the illumination beam path. Each sector can be turned by rotating filter draw 21 into the illumination beam path and cover it completely. The beam cross section with the diameter x is inscribed.

[0056] Each second sector is a blank aperture 22. Applied, for example glued on or vapor-deposited, in between each is one of the three filters 28, 29, 31 for the three excitation bands A, B, D. Each filter 28, 29, 31 exhibits an increase of the transmission factor inside its sector in one of the two directions of rotation, for example via different

surface coverage with vapor-deposited area elements. As a result each filter 28, 29, 31 has next to the one neighboring blank aperture 22 a maximum transmission factor and next to the other neighboring blank aperture 22 a minimum transmission factor. By rotating the filter draw 21, one of the blank apertures 22 or one of the filters 28, 29, 31 with the desired transmission or a combination of the two can be inserted in the illumination beam path. The use of two identical filter draws 21 as a filter draw assembly makes it possible to attenuate up to two of the three excitations bands A, B, D simultaneously and independently of each other to the desired setpoint value.

[0057] Figure 3d shows the spectral transmission curves of the short-wave excitation band A, of the long-wave excitation band D and an intermediate excitation band B as a function of the light wavelength λ .

[0058] Furthermore, the transmission curves T_A , T_B , T_D of the accompanying selective filters are depicted, thus the long-pass filter for the short-wave excitation band A, of the short-pass filter for the long-wave excitation band D and a selective filter for the excitation band B. The filters are selected so that they only selectively filter out the associated excitation band, but the remaining wavelength ranges pass through unhindered.

[0059] Figure 4a shows a circular filter draw 21 for a three-part filter draw assembly 20 of a multiband fluorescence microscope according to the invention having four excitation bands A, B, C, D. In this embodiment of the multiband fluorescence microscope according to the invention, filter draw assembly 20 consists of three circular filter draws identical to said bands that are tightly inserted in the illumination beam path one behind the other next to the aperture diaphragm plane 24. Each filter draw 21 has a blank aperture 22 in the center. In addition four selective filters 28, 29, 31, 32 for the excitation bands A, B, C, D, are vapor deposited as ring sectors that border each other.

[0060] The transmission factor of the filters 28, 29, 31, 32 diminishes as the radius increases and is largest next to the blank aperture 22. This is achieved, for example, by vapor-depositing the filters 28, 29, 31, 32 not completely, but as ring-shaped area

elements 30 with non-vapor-deposited areas with vapor-deposition between them, whereby the portion of the vapor-deposited area of each filter 28, 29, 31, 32 increases as the radius increases.

5 [0061] Separate positioning means (not shown) are assigned to each of the three filter draws 21 of filter draw assembly 20. Using these positioning means, each filter draw 21 can be moved individually and parallel to aperture diaphragm plane 24. In this case it can be shifted laterally in one direction and also rotated (as indicated in Figure 4a) or alternatively shifted in two directions within one plane.

10 [0062] As a result, any fractional area of filter draw 21, thus the blank aperture 22 or any fractional area filters 28, 29, 31, 32, can be inserted in the illumination beam path. By arranging three equal filter draws 21 in the illumination beam path, any three of the four filters 28, 29, 31, 32 can be simultaneously inserted in the illumination beam path. As a result up to three of the four excitation bands A, B, C, D can be weakened to the predetermined setpoint value or even faded out completely.

15 [0063] Figure 4b shows an especially advantageous embodiment of a filter draw 21 for a three-part filter draw assembly 20 for a multiband fluorescence microscope according to the invention having four excitation bands A, B, C, D. In this embodiment of the multiband fluorescence microscope according to the invention, filter draw assembly 20 consists of three circular filter draws 21 identical to said bands and are
20 inserted in the illumination beam path tightly in succession next to aperture diaphragm plane 24.

[0064] The filters 28, 29, 31, 32 for the excitation bands A, B, C, D are arranged on the filter draw 21 such that it only has to be rotated, but does not have to be moved (rotating movement is indicated). That simplifies the design of the mechanical or even
25 motorized positioning means that are separately assigned to each of the three filter draws 21 (not shown).

[0065] This is achieved by virtue of each filter draw 21 being designed with a circular shape, divided into eight sectors and mounted pivoted around its center. The center of the

circle is outside the illumination beam path. By rotating the filter draw 21, each sector can be brought to totally or partially cover the illumination beam path. The beam cross section with the diameter x is inscribed.

[0066] Every second sector is a blank aperture 22. In between them one of the four
 5 filters 28, 29, 31, 32 is applied, e.g., glued or vapor-deposited, for the three [sic] excitation bands A, B, C, D. Each filter 28, 29, 31, 32 exhibits an increase of the transmission factor inside its sector in one of the two directions of rotation, for example, via different area coverage. As a result each filter 28, 29, 31, 32 has a maximum transmission factor next to the one neighboring blank aperture 22 and a minimum
 10 transmission factor next to the other neighboring blank aperture 22.

[0067] By rotating filter draw 21, one of blank apertures 22 or one of filters 28, 29, 31, 32 having the desired transmission of a combination of both can be inserted in the illumination beam path. By using three filter draws 21, up to three of the four excitation bands A, B, C, D can be attenuated to the desired setpoint value simultaneously and
 15 independently of one another.

[0068] In Figure 4c, the spectral transmission curves of the short-wave excitation band A, of the long-wave excitation wave excitation band D and of the two excitation bands B and C between them are represented as a function of the light wavelength λ . Furthermore, the transmission curves T_A , T_B , T_C , T_D are specified for the associated
 20 selective filter, thus the long-pass filter for the short-wave excitation band A, the short-pass filter for the long-wave excitation band D, a selective filter for the excitation band B and a selective filter for the excitation band C. Filters A, B, C, D are selected so that they only selectively filter out the particular associated excitation band, but the remaining wavelength ranges pass through unhindered.

[0069] For more than four excitation bands, the filter draw assembly 20 must be
 25 expanded accordingly. However, it is becoming increasingly difficult to place the individual filters close enough next to aperture diaphragm 5.

Reference symbol list

	1	Light source
	2	Optical axis of the illumination beam path
	3	Collector
5	4	First lens member
	5	Aperture diaphragm
	6	Second lens member
	7	Radiant field stop
	8	Third lens member
10	9	Excitation filter
	10	Beam splitter
	11	Objective
	12	Fluorescence object
	13	Specimen stage
15	14	Output filter
	15	Tube lens
	16	Intermediate image plane
	17	Eyepiece on tube (17')
	18	Video camera
20	19	Image analysis system
	20	Filter draw assembly
	21	Filter draw (also 21a, 21b)
	22	Blank aperture
	23	Selective filter(s)
25	24	Aperture diaphragm plane
	25	Positioning means (25a, 25b motors)
	26	Control electronics
	27	Glass plate

	28	Short-pass filter for the long-wave excitation band D (λ_4)
	29	Long-pass filter for the short-wave excitation band A (λ_1)
	30	Area element(s)
	31	Subtractive filter for excitation B(λ_2)
5	32	Subtractive filter for excitation C(λ_3)
x		Beam diameter in the aperture diaphragm plane 24

WHAT IS CLAIMED IS:

TITLE OF THE INVENTION

Method for the Individual Adaptation of Excitation Intensities in a Multiband
Fluorescence Microscope and a Multiband Fluorescence Microscope for Carrying Out
Said Method

5

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application is the U.S. national phase under 35 U.S.C. 371 of
International Application No. PCT/DE99/03768 filed November 27, 1999 claiming
priority of German Patent Application No. 198 58 206.4 filed December 17, 1998.

10

FIELD OF THE INVENTION

[0002] The invention relates to a method for the individual adaptation of excitation
intensities in a multiband fluorescence microscope and a multiband fluorescence
microscope to execute the method corresponding to the features of the introductory clause
of the independent claims.

15

BACKGROUND OF THE INVENTION

[0003] With the multiband fluorescence microscope, the user frequently confronts the
problem that the different fluorescence bands in the microscopic image have varying
intensities and are not uniformly visible. The cause lies frequently in the difference in
excitation intensities in the illumination beam path or even in the varying blockage of the
fluorescence intensities by a barrier filter in the imaging beam path. Also, different
concentrations of the fluorescence die for the different excitation bands, even when
staining the objects to be considered, or the progressive bleaching of the dye, the so-
called fading, lead to differing intensities of the fluorescence bands in the microscopic
image. The different intensities of the fluorescence bands prove to be especially
problematic then if the microscopic image is to be photographically recorded. Then the

20

25

portion of the fluorescent light of weak intensity on the photo is too weakly reproduced or is not visible at all. Only with intensities of the fluorescence bands that are as uniform as possible can there be defect-free photos of the microscopic image.

[0004] US Patent 5,371,624 specifies a fluorescence microscope having only two
 5 excitation bands in which the intensities of the two excitation bands can be intermittently affected. It includes an illumination beam path having a light source and an excitation filter that produces several excitation bands of varying light wavelengths from the light of the light source. Furthermore, it has a splitter mirror, an output filter (also designated as a barrier filter or emission filter) for the fluorescent light and a filter element for affecting
 10 the intensities of the excitation bands.

[0005] The filter element can, by tilting continuously with respect to the optical axis, be switched between two limit positions having two fixed, predetermined values of the transmission factors of one or the other excitation band. In the one limit position, only the first excitation band is attenuated, in the other limit position only the second
 15 excitation band is attenuated and between the two limit positions, neither of the excitation bands is attenuated. A drop in the transmission factor of the particular excitation band is only possible up to the fixed, predetermined value. However, variation between a maximum transmission and a zero value, i.e., up to full suppression of one of the two excitation bands, is not possible. Moreover, only two excitation bands can be affected.

20

SUMMARY OF THE INVENTION

[0006] It is object of the present invention to specify a method for individual adaptation of excitation intensities in a multiband fluorescence microscope and a multiband fluorescence microscope to execute the method, in which one or more of the
 25 excitation bands can be partially or completely filtered out. For this purpose the transmission factor for each excitation band is to be continuously adjustable using simple means.

[0007] This objective is met by the invention via the characterizing features of the independent claims. Advantageous embodiments arise from the features of the dependent claims.

[0008] The method of the invention starts from a known multiband fluorescence microscope in which several excitation bands of varying wavelengths are produced using an excitation filter in an illumination beam path from the light of a light source. The excitation bands illuminate a fluorescence object prepared using fluorescence dyes and are converted by it into frequency-shifted fluorescence bands.

[0009] According to the invention, the fluorescence intensities of the individual fluorescence bands are first determined in the microscopic image and compared to previously set intensity setpoint values. The fluorescence intensities can be determined, for example, either visually or by using an intensity meter. This can consist for example of a video or CCD camera having an image analysis system connected in an outgoing circuit.

[0010] In this context a different intensity setpoint value can be set for each fluorescence band. However, in practice, the setpoint values are oriented toward concrete problems posed by the microscope user. If, for example, the microscopic image is to be documented either photographically or by video camera, and thus each fluorescence band in the photo or in the video image is to be reproduced with equal brightness, then the level of the setpoint values depends on the spectral sensitivity of the film or the camera. Therefore, their spectral sensitivity must be taken into consideration in the determination of setpoint values for the various excitation bands.

[0011] Therefore, the desired setpoint values must all be equal – and in particular equal to the lowest fluorescence intensity – provided that the film or the camera reproduces all spectral colors with equal intensity. However, if the spectral sensitivity of the video camera or of the film is not constant, then correspondingly varying setpoint values must be set for the different fluorescence bands in order to be able to reproduce the fluorescence bands with equal brightness.

[0012] On the other hand, if specific fluorescence bands do not appear on the photo or the video image, and thus are masked out, then the setpoint values for said bands must be equal to zero. In this context, it proves to be beneficial if the setpoint values are equal to the lowest of their intensities also for the fluorescence bands that are not masked out.

5 Then these fluorescence bands all appear equally bright.

[0013] For each excitation band that is assigned to a fluorescence intensity deviating from the setpoint values, a selective filter according to the present invention tuned to the pertinent excitation band is brought into the illumination beam path. Its spectral transmission curve is configured so that exclusively the intensity of the pertinent
10 excitation band is reduced, but the remaining spectral regions pass through unhindered.

[0014] In a multiband fluorescence microscope according to the invention for executing the method according to the invention, a filter draw assembly made of a multiplicity of individually movable filter draws is perpendicularly inserted in the illumination beam path tightly next to the aperture diaphragm plane.

15 **[0015]** The structure of the filter draw is a function of the number of the different excitation bands of the multiband fluorescence microscope. For a number of n excitation bands, each filter draw has n selective filters harmonized to the excitation bands and having surface regions with high and low transmission factors.

[0016] The different transmission factors are achieved by virtue of the fact that only
20 certain area portions of the beam cross-section are occupied using separate filter-area elements. In this context as even a surface coverage of the beam cross-section as possible is sought so that no unilateral shading, and thus also no unilateral illumination of the pupils, is made. As a result a crooked illumination, and thus a lateral migration of the picture elements, is prevented during focusing.

25 **[0017]** It must be possible to insert the filters in the illumination beam path independently of each other individually or in combination using the desired surface region or the desired transmission. In this context, it must be possible with n excitation bands to combine a maximum of $n-1$ filters with each other, i.e. simultaneously insert

them in the illumination beam path. This is sufficient since all excitation bands must never be attenuated simultaneously, because as a rule an excitation band supplies the intensity setpoint value and remains unchanged. Likewise, all excitation bands must not be triggered simultaneously, since this is equivalent to turning off the lighting.

5 **[0018]** In order to achieve the required combinations from $n-1$ filters, $n-1$ individually movable filter draws that are situated tightly parallel next to the aperture diaphragm plane with little distance between them and are effectual in combination are arranged in an advantageous embodiment of a multiband fluorescence microscope according to the invention having n excitation bands on $n-1$ disk planes. With each filter draw, one filter
10 for an excitation band can be suitably adjusted so that altogether a maximum of $n-1$ excitation bands (one less than the maximum number) can be selectively attenuated or triggered.

[0019] For two excitation bands, it is sufficient if the two required filters are arranged in a single disk plane ($n-1=1$), since either only the one or only the other filter must be
15 inserted in the illumination beam path. Therefore, a filter draw assembly to affect two excitation bands is preferably of one piece, thus designed with only one filter draw. However, for more than two excitation bands, a corresponding multi-piece filter draw assembly must be provided with $n-1$ individually movable filter draws.

[0020] Except for the selective filters for the various excitation bands, each filter
20 draw has at least one blank aperture with the beam diameter of the illumination beam path. In this case a blank aperture is arranged next to each filter. Depending on the embodiment, the filters can also be grouped directly around a single blank aperture. The transmission factor of the filter diminishes in the shifting direction as the distance from the blank aperture increases. In this case each filter has a surface region with the lowest
25 transmission factor and a minimum diameter equal to the beam diameter x . If this surface region is inserted in the illumination beam path, the associated excitation band is maximally attenuated, i.e., to the zero value.

[0021] By correctly positioning each of the individual filters in the illumination beam path, the transmission factor that is acting on the illumination beam path is precisely individually adjusted such that by attenuating the excitation band assigned to the filter, the resulting fluorescence intensity matches its intensity setpoint value. To execute this process step, separate positioning means, which bring about the full or partial covering of the illumination beam path using the appropriate surface region of the filter draw, are assigned to each filter draw. Thus, the blank aperture or one or more of the filters or combinations of filter regions and the blank aperture can be inserted in the illumination beam path, according to preference. If this last process step was executed for all filters and thus all excitation bands, all fluorescence intensities match their setpoint values.

[0022] Even after optimal setting of the fluorescence intensities by applying the method according to the invention using a specified multiband fluorescence microscope, deviations of the fluorescence intensities from the setpoint values reoccur after some time. This is attributable to the fact that the various fluorescence dyes for the various excitation bands fade at differing rates, i.e., they exhibit a specific fading.

[0023] Therefore, in one advantageous embodiment of the method, the time changes of the fluorescence intensities are continuously determined. The transmission factors of the filter that are in effect within the illumination beam path are then repeatedly adjusted at specific time intervals so that the fluorescence intensities are always brought into accord with their setpoint values.

[0024] An especially advantageous embodiment of the method provides for an automatic fading compensation. For this purpose the time modifications of the fluorescence intensities are automatically continually determined. To do this the microscopic image, for example, can be automatically continually recorded using a video camera and the fluorescence intensities contained therein can be determined using an image analysis system and compared to the predetermined setpoint values.

[0025] Then the transmission factors of the filter are automatically continuously modified and adapted so that the fluorescence intensities are always kept at the intensity

setpoint values. To this end, motorized positioning means, for example, can be used. The drive of the motors and regulation of the fluorescence intensities in relation to the setpoint values can be achieved using an electronic unit and a computer to which the signals of the video camera or the image analysis system are fed.

5

BRIEF DESCRIPTION OF THE DRAWINGS

[0026] The invention is explained in more detail below with respect to the schematic drawing. Shown are:

- Figure 1 a beam path of a multiband fluorescence microscope according to the invention;
- Figure 2a-d various embodiments of a filter draw for a dual-band fluorescence microscope;
- Figure 2e the spectral transmission curve of the excitation bands and the assigned filter of a filter draw for a dual-band fluorescence microscope;
- Figure 3a-c various embodiments of a filter draw for a two-piece filter draw assembly of a three-band fluorescence microscope;
- Figure 3d the spectral transmission curve of the excitation bands and the assigned filter of a filter draw for a three-band fluorescence microscope;
- Figure 4a-b various embodiments of a filter draw for a three-part filter draw assembly of a four-band fluorescence microscope;
- Figure 4c the spectral transmission curve of the excitation bands and the assigned filter of a filter draw for a four-band fluorescence microscope.

DETAILED DESCRIPTION OF THE INVENTION

- [0027]** Figure 1 shows a beam path of a multiband fluorescence microscope for executing the method according to the invention. From a light source 1 an illumination beam path starts with an optical axis 2 in which a collector 3, a first lens member 4, an aperture diaphragm 5, a second lens member 6, a radiant field stop 7, a third lens member

8 and an excitation filter 9 are arranged in succession to produce the excitation bands. The illumination beam path is deflected at a beam splitter 10 to an objective 11. It passes through the objective 11 and reaches a fluorescence object 12, which rests on a specimen stage 13.

5 **[0028]** The excitation bands produced by excitation filter 9 in the illumination beam path are converted into fluorescence bands 12 by fluorescence dyes that were injected in fluorescence object 12, and said bands are emitted by the fluorescence object in a frequency-shifted state. This fluorescence light passes through objective 11, beam splitter 10, an output filter 14, a tube lens 15 and reaches an intermediate image plane 16.

10 The intermediate image produced here can be considered by microscope user using an eyepiece 17. The intermediate image is displayed via a TV output on a video camera 18 having an image analysis system 19 connected in series. With this design the fluorescence intensities of the individual fluorescence bands in the microscopic image can be determined either visually using eyepiece 17 or using image analysis system 19
15 and then compared to intensity setpoint values greater than or equal to zero.

[0029] To attenuate the excitation bands, a filter draw assembly 20 according to the invention, consisting in this example of two filter draws 21a and 21b, is inserted perpendicular to the optical axis 2 of the illumination beam path tightly next to, i.e., behind or in front of aperture diaphragm plane 24. Arranged on each filter draw 21a, 21b
20 are a blank aperture 22 and in addition several selective filters 23 tuned to the excitation bands. By arranging filter draws 21a, 21b as narrowly as possible, filters 23 can be situated as tightly as possible next to aperture diaphragm plane 24. This ensures that the inserted filter draws 21a, 21b are not visible in the image. A further advantage is that in this case the least possible beam fanning occurs, so that the surface region of filter 23 can
25 be kept as small as possible.

[0030] According to the present invention, positioning means are assigned to each filter draw. In this context positioning means having the possibility of manual adjustability can be used in economic embodiments. Although motorized positioning

means are more expensive, they permit an automation of the method according to the invention.

- [0031]** In the embodiment illustrated here, two motors 25a, 25b that can shift parallel to the aperture diaphragm plane and/or rotate filter draws 21a, 21b are arranged as
- 5 positioning means for filter draws 21a, 21b. Using the motors 25a, 25b either the blank aperture 22 or a selective filter 23 or a combination of the two can be inserted in the illumination beam path by the particular assigned filter draw 21a or 21b. The motors 25a, 25b are driven by control electronics 26, which receive the input signal from the image analysis system 19.
- 10 **[0032]** The image analysis system 19 determines from the camera signal the deviation of the fluorescence intensities from their setpoint values. Then those excitation bands that cause the deviations from setpoint values are determined and one of the filter draws 21a, 21b is assigned to each of these excitation bands for attenuation. In the illustrated example, two excitation bands can thus be completely attenuated, i.e. to the zero value, or
- 15 even partially attenuated. For each filter draw 21a, 21b a drive signal is produced for the associated motor 25a, 25b. Each filter draw 21a, 21b is continuously shifted via the associated motor 25a, 25b so that a filter 23 selectively acting on the assigned excitation band is inserted in the illumination beam path. Then the filter draw 21a or 21b is shifted further until a surface region of the inserted filter 23 has a transmission factor in the
- 20 illumination beam path through which the assigned excitation band is attenuated to the intensity setpoint value. In this way, all excitation bands are set to their intensity setpoint values.

- [0033]** Different embodiments of filter draw assembly 20 or the filter draw 21 and the associated spectral transmission curves of the filters arranged on them are explained
- 25 below.

[0034] A filter draw 21 for a multiband fluorescence microscope having two excitation bands A and D is depicted in Figure 2a. Applied to a rectangular glass plate 27 as different vapor deposition layers are a long-pass filter 29 for the intensity reduction of

the short-wave excitation band A and a short-pass filter 28 for the intensity reduction of the long-wave excitation band D. They are located on the ends of the glass plate 27, and between them is the blank aperture 22 in the center of filter draw 21. Its cross-section is equal to the beam diameter x in the aperture diaphragm 5 of the illumination beam path.

- 5 Filter draw 21 can be continuously shifted by positioning means (not shown) longitudinally in both directions parallel to the aperture diaphragm plane 24. The movement is indicated by a double arrow.

[0035] Short-pass filter 28 and long-pass filter 29 are each applied in this version as a connected vapor-deposition layer. The transmission of the two filters 28, 29 is largest right next to blank aperture 22 and gets smaller as the distance from blank aperture 22 increases. To this end next to blank aperture 22 filter draw 21 is not completely covered with filters 28, 29, but rather the portion of the filter area increases as one moves from blank aperture 22 toward the end of filter draw 21.

[0036] Thus, the vapor-deposition layers on the ends of filter draw 21 form a rectangle with a minimum edge length equal to the beam diameter x . If this vapor-deposited rectangular area completely covers the illumination beam path, the smallest percentile is thus set and the accompanying excitation band is completely attenuated, i.e., to the zero value. The vapor-deposited rectangular area borders the base of vapor-deposited triangular area, whose opposing corner faces blank aperture 22.

20 **[0037]** Via the vapor-deposited triangular areas, which always cover the beam diameter only partially, the transmission factor of short-pass filter 28 and long-pass filter 29 in each case diminishes as one moves from blank aperture 22 toward the ends of filter draw 21. By inserting any fractional area of short-pass filter 28 or of long-pass filter 29, any desired transmission factor of the filter 28, 29 in the illumination beam path can be realized and thus one or the other excitation band can be weakened or faded out according to preference.

[0038] A filter draw 21 for a multiband fluorescence microscope according to the invention having two excitation bands A and D is likewise depicted in Figure 2b.

Applied to a rectangular glass plate 27 are a short-pass filter 28 for filtering the long-wave excitation band A and a long-pass filter 29 for filtering the short-wave excitation band D as different vapor-deposition layers.

5 **[0039]** Here, as in Figure 2a, the vapor-deposition layers in the direction of blank aperture 22 have a triangular contour. However, the triangles in this case are each not vapor-deposited as connected areas, but rather as small area elements 30 of equal size. In this way a longer adjustment range with higher transmissions is achieved for the two filters 28, 29. At the ends of filter draw 21, the vapor-deposition layers form a rectangle having a minimum edge length equal to the beam diameter x and thus a region of the least
10 transmission with which the assigned excitation band can be attenuated to zero.

15 **[0040]** Another filter draw 21 for a multiband fluorescence microscope according to the invention having two excitation bands A and D is depicted in Figure 2c. Applied to a rectangular glass plate 27 are a short-pass filter 28 for filtering the long-wave excitation band A and a long-pass filter 29 for filtering the short-wave excitation band D as different vapor-deposition layers. Located between them is a blank aperture 22.

20 **[0041]** The filters 28, 29 are vapor-deposited in this version of filter draw 21 next to blank aperture 22 as separate area elements 30 formed in any way desired (here circular).

25 **[0042]** In this context, the size of area elements 30 and thus the portion of the area of filter draw 21 increases when moving from blank aperture 22 to the ends of filter draw 21. The transmission factor of filters 28, 29 is largest next to blank aperture 22 and diminishes when moving in the direction toward the ends of filter draw 21. On both ends of filter draw 21, each of the two filters 28, 29 have a completely vapor-deposited area with the smallest transmission factor, said area having at least the diameter x of the beam path.

30 **[0043]** Another filter draw 21 for a multiband fluorescence microscope according to the invention having two excitation bands A and D is depicted in Figure 2d. Applied to a rectangular glass plate 27 are a short-pass filter 28 for filtering the long-wave excitation

band A and a long-pass filter 29 for filtering the short-wave excitation band D as different vapor-deposition layers. Located between them is a blank aperture 22.

5 **[0044]** In this embodiment the vapor-deposition layers are applied as strip-shaped area elements 30, the width of the strips increasing as one moves from the center to the ends of filter draw 21. In this way the transmission diminishes as one moves from the blank aperture 22 toward the ends of filter draw 21. Also here the two filters 28, 29 each have a completely vapor-deposited surface with the lowest possible transmission factor at both ends of filter draw 21, said surface having at least the diameter x of the beam path.

10 **[0045]** The spectral transmission curves of the short-wave excitation band A and the long-wave excitation band D, as well as the transmission curves T_A , T_D of the associated selective filter, thus the long-pass filter for the short-wave excitation band A and the short-pass filter for the long-wave excitation band D, are depicted in Figure 2e as a function of the light wavelength λ . Filters A and B are selected so that they only selectively filter out the associated excitation band for each but allow the remaining
15 wavelength ranges to pass through unhindered.

[0046] Figure 3a shows a rectangular filter draw 21 of a two-piece filter draw assembly 20 for a multiband fluorescence microscope having three excitation bands A, B, D. In this embodiment filter draw assembly 20 consists of two rectangular filter draws 21 identical to said bands that are tightly inserted in the illumination beam path one behind
20 the other next to the aperture diaphragm plane 24.

[0047] Provided in the center of filter draw 21 is the blank aperture 22 having diameter x of illumination beam path 2. Applied as vapor-deposition layers on the two ends of filter draw 21 are two different combinations of two or three selective filters 28, 29, 31 for excitation bands A, B, D. The two filters 28, 31 or 29, 31 on one end are in
25 each case arranged next to one another so that both filters border each other and blank aperture 22. In this context each filter 28, 29, 31 has an area of least transmission having at least the diameter x of illumination beam path 2. As a result filter draw 21 has at least a width of $2x$ on its short end.

[0048] The transmission factor of filters 28, 29, 31 diminishes as one moves in the longitudinal direction of filter draw 21, thus in the direction of shift from blank aperture 22 toward the ends. This is realized in the example represented here in that the vapor-deposited surface of each filter 28, 29, 31 increases as one moves from blank aperture 22 toward the end of filter draw 21.

[0049] Separate positioning means 25 for shifting filter draw 21 parallel to aperture diaphragm plane 24 in both the longitudinal and transverse directions are provided for each of the two identical shifters 21 of the filter draw assembly. As a result it is possible to insert filters 28, 29, 31, individually or in combination, completely or with only a portion of their surface in the illumination beam path.

[0050] Figure 3b shows a circular filter draw 21 of a two-piece filter draw assembly 20 for a multiband fluorescence microscope according to the invention having three excitation bands A, B, D. In this advantageous embodiment of the multiband fluorescence microscope according to the invention, filter draw assembly 20 consists of two circular filter draws 21 identical to said bands that are tightly inserted in the illumination beam path one behind the other next to the aperture diaphragm plane 24. Provided in the center of each filter draw 21 is blank aperture 22. Three selective filters 28, 29, 31 for excitation bands A, B, D are arranged bordering said opening as vapor-deposited ring sectors that border each other.

[0051] Filters 28, 29, 31 each possess a transmission factor that decreases as radius increases and is largest next to blank aperture 22. This is accomplished, for example, by virtue of filters 28, 29, 31 not being fully vapor-deposited. Instead, vapor-deposited area elements 30 that get wider as they extend radially and that have non-vapor-deposited surface regions are applied in between them next to blank aperture 22 so that the portion of vapor-deposited surface of each filter 28, 29, 31 increases as the radius increases.

[0052] Using separate positioning means (not shown here), each of the two identical filter draws 21 of filter draw assembly 20 can be shifted individually and parallel to aperture diaphragm plane 24. In this case it can either be laterally shifted in one direction

and also rotated (as indicated in Figure 3b) or, alternatively, shifted within a plane in two directions. As a result any desired fractional area of filter draw 21, for example blank aperture 22 or one of the filters 28, 29, 31 having the desired transmission factor, can be inserted in the illumination beam path. By arranging two equal filter draws 21 in the illumination beam path, any two of the three filters 28, 29, 31 can be inserted simultaneously in the illumination beam path so that two of the three excitation bands can be simultaneously weakened to the predetermined setpoint values or completely faded out.

[0053] Figure 3c shows an especially advantageous embodiment of a filter draw 21 for a two-piece filter draw assembly 20 of a multiband fluorescence microscope according to the invention having three excitation bands A, B, D. In this embodiment of the multiband fluorescence microscope, filter draw assembly 20 consists of two circular filter draws 21 identical to said bands that are inserted in the illumination beam path tightly in succession next to aperture diaphragm plane 24.

[0054] The filters 28, 29, 31 for the excitation bands A, B, D are advantageously arranged on the filter draw 21 such that it only has to be turned but not shifted (rotary movement is indicated). This simplifies the structure of the mechanical or even motorized positioning means, which are separately assigned to each of the two filter draws 21 (not shown).

[0055] In order to accomplish this, each filter draw 21 is designed with a circular shape, divided into six sectors and arranged pivoted around its center. The center of the circle lies outside the illumination beam path. Each sector can be turned by rotating filter draw 21 into the illumination beam path and cover it completely. The beam cross section with the diameter x is inscribed.

[0056] Each second sector is a blank aperture 22. Applied, for example glued on or vapor-deposited, in between each is one of the three filters 28, 29, 31 for the three excitation bands A, B, D. Each filter 28, 29, 31 exhibits an increase of the transmission factor inside its sector in one of the two directions of rotation, for example via different

surface coverage with vapor-deposited area elements. As a result each filter 28, 29, 31 has next to the one neighboring blank aperture 22 a maximum transmission factor and next to the other neighboring blank aperture 22 a minimum transmission factor. By rotating the filter draw 21, one of the blank apertures 22 or one of the filters 28, 29, 31 with the desired transmission or a combination of the two can be inserted in the illumination beam path. The use of two identical filter draws 21 as a filter draw assembly makes it possible to attenuate up to two of the three excitations bands A, B, D simultaneously and independently of each other to the desired setpoint value.

[0057] Figure 3d shows the spectral transmission curves of the short-wave excitation band A, of the long-wave excitation band D and an intermediate excitation band B as a function of the light wavelength λ .

[0058] Furthermore, the transmission curves T_A , T_B , T_D of the accompanying selective filters are depicted, thus the long-pass filter for the short-wave excitation band A, of the short-pass filter for the long-wave excitation band D and a selective filter for the excitation band B. The filters are selected so that they only selectively filter out the associated excitation band, but the remaining wavelength ranges pass through unhindered.

[0059] Figure 4a shows a circular filter draw 21 for a three-part filter draw assembly 20 of a multiband fluorescence microscope according to the invention having four excitation bands A, B, C, D. In this embodiment of the multiband fluorescence microscope according to the invention, filter draw assembly 20 consists of three circular filter draws identical to said bands that are tightly inserted in the illumination beam path one behind the other next to the aperture diaphragm plane 24. Each filter draw 21 has a blank aperture 22 in the center. In addition four selective filters 28, 29, 31, 32 for the excitation bands A, B, C, D, are vapor deposited as ring sectors that border each other.

[0060] The transmission factor of the filters 28, 29, 31, 32 diminishes as the radius increases and is largest next to the blank aperture 22. This is achieved, for example, by vapor-depositing the filters 28, 29, 31, 32 not completely, but as ring-shaped area

elements 30 with non-vapor-deposited areas with vapor-deposition between them, whereby the portion of the vapor-deposited area of each filter 28, 29, 31, 32 increases as the radius increases.

5 **[0061]** Separate positioning means (not shown) are assigned to each of the three filter draws 21 of filter draw assembly 20. Using these positioning means, each filter draw 21 can be moved individually and parallel to aperture diaphragm plane 24. In this case it can be shifted laterally in one direction and also rotated (as indicated in Figure 4a) or alternatively shifted in two directions within one plane.

10 **[0062]** As a result, any fractional area of filter draw 21, thus the blank aperture 22 or any fractional area filters 28, 29, 31, 32, can be inserted in the illumination beam path. By arranging three equal filter draws 21 in the illumination beam path, any three of the four filters 28, 29, 31, 32 can be simultaneously inserted in the illumination beam path. As a result up to three of the four excitation bands A, B, C, D can be weakened to the predetermined setpoint value or even faded out completely.

15 **[0063]** Figure 4b shows an especially advantageous embodiment of a filter draw 21 for a three-part filter draw assembly 20 for a multiband fluorescence microscope according to the invention having four excitation bands A, B, C, D. In this embodiment of the multiband fluorescence microscope according to the invention, filter draw assembly 20 consists of three circular filter draws 21 identical to said bands and are
20 inserted in the illumination beam path tightly in succession next to aperture diaphragm plane 24.

[0064] The filters 28, 29, 31, 32 for the excitation bands A, B, C, D are arranged on the filter draw 21 such that it only has to be rotated, but does not have to be moved (rotating movement is indicated). That simplifies the design of the mechanical or even
25 motorized positioning means that are separately assigned to each of the three filter draws 21 (not shown).

[0065] This is achieved by virtue of each filter draw 21 being designed with a circular shape, divided into eight sectors and mounted pivoted around its center. The center of the

circle is outside the illumination beam path. By rotating the filter draw 21, each sector can be brought to totally or partially cover the illumination beam path. The beam cross section with the diameter x is inscribed.

5 **[0066]** Every second sector is a blank aperture 22. In between them one of the four filters 28, 29, 31, 32 is applied, e.g., glued or vapor-deposited, for the three [sic] excitation bands A, B, C, D. Each filter 28, 29, 31, 32 exhibits an increase of the transmission factor inside its sector in one of the two directions of rotation, for example, via different area coverage. As a result each filter 28, 29, 31, 32 has a maximum transmission factor next to the one neighboring blank aperture 22 and a minimum
10 transmission factor next to the other neighboring blank aperture 22.

[0067] By rotating filter draw 21, one of blank apertures 22 or one of filters 28, 29, 31, 32 having the desired transmission of a combination of both can be inserted in the illumination beam path. By using three filter draws 21, up to three of the four excitation bands A, B, C, D can be attenuated to the desired setpoint value simultaneously and
15 independently of one another.

[0068] In Figure 4c, the spectral transmission curves of the short-wave excitation band A, of the long-wave excitation wave excitation band D and of the two excitation bands B and C between them are represented as a function of the light wavelength λ . Furthermore, the transmission curves T_A , T_B , T_C , T_D are specified for the associated
20 selective filter, thus the long-pass filter for the short-wave excitation band A, the short-pass filter for the long-wave excitation band D, a selective filter for the excitation band B and a selective filter for the excitation band C. Filters A, B, C, D are selected so that they only selectively filter out the particular associated excitation band, but the remaining wavelength ranges pass through unhindered.

25 **[0069]** For more than four excitation bands, the filter draw assembly 20 must be expanded accordingly. However, it is becoming increasingly difficult to place the individual filters close enough next to aperture diaphragm 5.

Reference symbol list

	1	Light source
	2	Optical axis of the illumination beam path
	3	Collector
5	4	First lens member
	5	Aperture diaphragm
	6	Second lens member
	7	Radiant field stop
	8	Third lens member
10	9	Excitation filter
	10	Beam splitter
	11	Objective
	12	Fluorescence object
	13	Specimen stage
15	14	Output filter
	15	Tube lens
	16	Intermediate image plane
	17	Eyepiece on tube (17')
	18	Video camera
20	19	Image analysis system
	20	Filter draw assembly
	21	Filter draw (also 21a, 21b)
	22	Blank aperture
	23	Selective filter(s)
25	24	Aperture diaphragm plane
	25	Positioning means (25a, 25b motors)
	26	Control electronics
	27	Glass plate

28	Short-pass filter for the long-wave excitation band D (λ_4)
29	Long-pass filter for the short-wave excitation band A (λ_1)
30	Area element(s)
31	Subtractive filter for excitation B(λ_2)
5 32	Subtractive filter for excitation C(λ_3)
x	Beam diameter in the aperture diaphragm plane 24

WHAT IS CLAIMED IS: [Claims]

4/PR17

09/868129

JC03 Rec'd PCT/PTC

14 JUN 2001

Express Mail Label No. EL917291338US

LWEP:103_US_

Translation of International Application PCT/DE99/03768

Method for the Individual Adaptation of Excitation Intensities in a Multiband
Fluorescence Microscope and a Multiband Fluorescence Microscope for Carrying Out
Said Method

- 5 The invention relates to a method for the individual adaptation of excitation intensities in a multiband fluorescence microscope and a multiband fluorescence microscope to execute the method corresponding to the features of the introductory clause of the independent claims.
- 10 With the multiband fluorescence microscope, the user frequently confronts the problem that the different fluorescence bands in the microscopic image have varying intensities and are not uniformly visible. The cause lies frequently in the difference in excitation intensities in the illumination beam path or even in the varying blockage of the fluorescence intensities by a barrier filter in the imaging beam path. Also, different
- 15 concentrations of the fluorescence die for the different excitation bands, even when staining the objects to be considered, or the progressive bleaching of the dye, the so-called fading, lead to differing intensities of the fluorescence bands in the microscopic image. The different intensities of the fluorescence bands prove to be especially problematic then if the microscopic image is to be photographically recorded. Then the
- 20 portion of the fluorescent light of weak intensity on the photo is too weakly reproduced or is not visible at all. Only with intensities of the fluorescence bands that are as uniform as possible can there be defect-free photos of the microscopic image.

- US Patent 5,371,624 specifies a fluorescence microscope having only two excitation
- 25 bands in which the intensities of the two excitation bands can be intermittently affected. It includes an illumination beam path having a light source and an excitation filter that produces several excitation bands of varying light wavelengths from the light of the light source. Furthermore, it has a splitter mirror, an output filter (also designated as a barrier

filter or emission filter) for the fluorescent light and a filter element for affecting the intensities of the excitation bands.

5 The filter element can, by tilting continuously with respect to the optical axis, be switched between two limit positions having two fixed, predetermined values of the transmission factors of one or the other excitation band. In the one limit position, only the first excitation band is attenuated, in the other limit position only the second excitation band is attenuated and between the two limit positions, neither of the excitation bands is attenuated. A drop in the transmission factor of the particular excitation band is only
10 possible up to the fixed, predetermined value. However, variation between a maximum transmission and a zero value, i.e., up to full suppression of one of the two excitation bands, is not possible. Moreover, only two excitation bands can be affected.

15 It is object of the present invention to specify a method for individual adaptation of excitation intensities in a multiband fluorescence microscope and a multiband fluorescence microscope to execute the method, in which one or more of the excitation bands can be partially or completely filtered out. For this purpose the transmission factor for each excitation band is to be continuously adjustable using simple means.

20 This objective is met by the invention via the characterizing features of the independent claims. Advantageous embodiments arise from the features of the dependent claims.

The method of the invention starts from a known multiband fluorescence microscope in which several excitation bands of varying wavelengths are produced using an excitation
25 filter in an illumination beam path from the light of a light source. The excitation bands

illuminate a fluorescence object prepared using fluorescence dyes and are converted by it into frequency-shifted fluorescence bands.

According to the invention, the fluorescence intensities of the individual fluorescence

5 bands are first determined in the microscopic image and compared to previously set intensity setpoint values. The fluorescence intensities can be determined, for example, either visually or by using an intensity meter. This can consist for example of a video or CCD camera having an image analysis system connected in an outgoing circuit.

10 In this context a different intensity setpoint value can be set for each fluorescence band. However, in practice, the setpoint values are oriented toward concrete problems posed by the microscope user. If, for example, the microscopic image is to be documented either photographically or by video camera, and thus each fluorescence band in the photo or in the video image is to be reproduced with equal brightness, then the level of the setpoint

15 values depends on the spectral sensitivity of the film or the camera. Therefore, their spectral sensitivity must be taken into consideration in the determination of setpoint values for the various excitation bands.

Therefore, the desired setpoint values must all be equal – and in particular equal to the

20 lowest fluorescence intensity – provided that the film or the camera reproduces all spectral colors with equal intensity. However, if the spectral sensitivity of the video camera or of the film is not constant, then correspondingly varying setpoint values must be set for the different fluorescence bands in order to be able to reproduce the fluorescence bands with equal brightness.

25

On the other hand, if specific fluorescence bands do not appear on the photo or the video image, and thus are masked out, then the setpoint values for said bands must be equal to zero. In this context, it proves to be beneficial if the setpoint values are equal to the lowest of their intensities also for the fluorescence bands that are not masked out. Then
5 these fluorescence bands all appear equally bright.

For each excitation band that is assigned to a fluorescence intensity deviating from the setpoint values, a selective filter according to the present invention tuned to the pertinent excitation band is brought into the illumination beam path. Its spectral transmission curve
10 is configured so that exclusively the intensity of the pertinent excitation band is reduced, but the remaining spectral regions pass through unhindered.

In a multiband fluorescence microscope according to the invention for executing the method according to the invention, a filter draw assembly made of a multiplicity of
15 individually movable filter draws is perpendicularly inserted in the illumination beam path tightly next to the aperture diaphragm plane.

The structure of the filter draw is a function of the number of the different excitation bands of the multiband fluorescence microscope. For a number of n excitation bands,
20 each filter draw has n selective filters harmonized to the excitation bands and having surface regions with high and low transmission factors.

The different transmission factors are achieved by virtue of the fact that only certain area portions of the beam cross-section are occupied using separate filter-area elements. In this
25 context as even a surface coverage of the beam cross-section as possible is sought so that no unilateral shading, and thus also no unilateral illumination of the pupils, is made. As a

result a crooked illumination, and thus a lateral migration of the picture elements, is prevented during focusing.

It must be possible to insert the filters in the illumination beam path independently of each other individually or in combination using the desired surface region or the desired transmission. In this context, it must be possible with n excitation bands to combine a maximum of $n-1$ filters with each other, i.e. simultaneously insert them in the illumination beam path. This is sufficient since all excitation bands must never be attenuated simultaneously, because as a rule an excitation band supplies the intensity setpoint value and remains unchanged. Likewise, all excitation bands must not be triggered simultaneously, since this is equivalent to turning off the lighting.

In order to achieve the required combinations from $n-1$ filters, $n-1$ individually movable filter draws that are situated tightly parallel next to the aperture diaphragm plane with little distance between them and are effectual in combination are arranged in an advantageous embodiment of a multiband fluorescence microscope according to the invention having n excitation bands on $n-1$ disk planes. With each filter draw, one filter for an excitation band can be suitably adjusted so that altogether a maximum of $n-1$ excitation bands (one less than the maximum number) can be selectively attenuated or triggered.

For two excitation bands, it is sufficient if the two required filters are arranged in a single disk plane ($n-1=1$), since either only the one or only the other filter must be inserted in the illumination beam path. Therefore, a filter draw assembly to affect two excitation bands is preferably of one piece, thus designed with only one filter draw. However, for

more than two excitation bands, a corresponding multi-piece filter draw assembly must be provided with $n-1$ individually movable filter draws.

Except for the selective filters for the various excitation bands, each filter draw has at least one blank aperture with the beam diameter of the illumination beam path. In this case a blank aperture is arranged next to each filter. Depending on the embodiment, the filters can also be grouped directly around a single blank aperture. The transmission factor of the filter diminishes in the shifting direction as the distance from the blank aperture increases. In this case each filter has a surface region with the lowest transmission factor and a minimum diameter equal to the beam diameter x . If this surface region is inserted in the illumination beam path, the associated excitation band is maximally attenuated, i.e., to the zero value.

By correctly positioning each of the individual filters in the illumination beam path, the transmission factor that is acting on the illumination beam path is precisely individually adjusted such that by attenuating the excitation band assigned to the filter, the resulting fluorescence intensity matches its intensity setpoint value. To execute this process step, separate positioning means, which bring about the full or partial covering of the illumination beam path using the appropriate surface region of the filter draw, are assigned to each filter draw. Thus, the blank aperture or one or more of the filters or combinations of filter regions and the blank aperture can be inserted in the illumination beam path, according to preference. If this last process step was executed for all filters and thus all excitation bands, all fluorescence intensities match their setpoint values.

Even after optimal setting of the fluorescence intensities by applying the method according to the invention using a specified multiband fluorescence microscope,

deviations of the fluorescence intensities from the setpoint values reoccur after some time. This is attributable to the fact that the various fluorescence dyes for the various excitation bands fade at differing rates, i.e., they exhibit a specific fading.

5 Therefore, in one advantageous embodiment of the method, the time changes of the fluorescence intensities are continuously determined. The transmission factors of the filter that are in effect within the illumination beam path are then repeatedly adjusted at specific time intervals so that the fluorescence intensities are always brought into accord with their setpoint values.

10

An especially advantageous embodiment of the method provides for an automatic fading compensation. For this purpose the time modifications of the fluorescence intensities are automatically continually determined. To do this the microscopic image, for example, can be automatically continually recorded using a video camera and the fluorescence

15 intensities contained therein can be determined using an image analysis system and compared to the predetermined setpoint values.

Then the transmission factors of the filter are automatically continuously modified and adapted so that the fluorescence intensities are always kept at the intensity setpoint

20 values. To this end, motorized positioning means, for example, can be used. The drive of the motors and regulation of the fluorescence intensities in relation to the setpoint values can be achieved using an electronic unit and a computer to which the signals of the video camera or the image analysis system are fed.

25 The invention is explained in more detail below with respect to the schematic drawing. Shown are:

- Figure 1 a beam path of a multiband fluorescence microscope according to the invention;
- 5 Figure 2a-d various embodiments of a filter draw for a dual-band fluorescence microscope;
- Figure 2e the spectral transmission curve of the excitation bands and the assigned filter of a filter draw for a dual-band fluorescence microscope;
- 10 Figure 3a-c various embodiments of a filter draw for a two-piece filter draw assembly of a three-band fluorescence microscope;
- Figure 3d the spectral transmission curve of the excitation bands and the assigned filter of a filter draw for a three-band fluorescence microscope;
- 15 Figure 4a-b various embodiments of a filter draw for a three-part filter draw assembly of a four-band fluorescence microscope;
- 20 Figure 4c the spectral transmission curve of the excitation bands and the assigned filter of a filter draw for a four-band fluorescence microscope.

Figure 1 shows a beam path of a multiband fluorescence microscope for executing the method according to the invention. From a light source 1 an illumination beam path starts with an optical axis 2 in which a collector 3, a first lens member 4, an aperture diaphragm 5, a second lens member 6, a radiant field stop 7, a third lens member 8 and a excitation

25

filter 9 are arranged in succession to produce the excitation bands. The illumination beam path is deflected at a beam splitter 10 to an objective 11. It passes through the objective 11 and reaches a fluorescence object 12, which rests on a specimen stage 13.

- 5 The excitation bands produced by excitation filter 9 in the illumination beam path are converted into fluorescence bands 12 by fluorescence dyes that were injected in fluorescence object 12, and said bands are emitted by the fluorescence object in a frequency-shifted state. This fluorescence light passes through objective 11, beam splitter 10, an output filter 14, a tube lens 15 and reaches an intermediate image plane 16. The
10 intermediate image produced here can be considered by microscope user using an eyepiece 17. The intermediate image is displayed via a TV output on a video camera 18 having an image analysis system 19 connected in series. With this design the fluorescence intensities of the individual fluorescence bands in the microscopic image can be determined either visually using eyepiece 17 or using image analysis system 19 and then
15 compared to intensity setpoint values greater than or equal to zero.

- To attenuate the excitation bands, a filter draw assembly 20 according to the invention, consisting in this example of two filter draws 21a and 21b, is inserted perpendicular to the optical axis 2 of the illumination beam path tightly next to, i.e., behind or in front of
20 aperture diaphragm plane 24. Arranged on each filter draw 21a, 21b are a blank aperture 22 and in addition several selective filters 23 tuned to the excitation bands. By arranging filter draws 21a, 21b as narrowly as possible, filters 23 can be situated as tightly as possible next to aperture diaphragm plane 24. This ensures that the inserted filter draws 21a, 21b are not visible in the image. A further advantage is that in this case the least
25 possible beam fanning occurs, so that the surface region of filter 23 can be kept as small as possible.

According to the present invention, positioning means are assigned to each filter draw. In this context positioning means having the possibility of manual adjustability can be used in economic embodiments. Although motorized positioning means are more expensive,
5 they permit an automation of the method according to the invention.

In the embodiment illustrated here, two motors 25a, 25b that can shift parallel to the aperture diaphragm plane and/or rotate filter draws 21a, 21b are arranged as positioning means for filter draws 21a, 21b. Using the motors 25a, 25b either the blank aperture 22 or
10 a selective filter 23 or a combination of the two can be inserted in the illumination beam path by the particular assigned filter draw 21a or 21b. The motors 25a, 25b are driven by control electronics 26, which receive the input signal from the image analysis system 19.

The image analysis system 19 determines from the camera signal the deviation of the
15 fluorescence intensities from their setpoint values. Then those excitation bands that cause the deviations from setpoint values are determined and one of the filter draws 21a, 21b is assigned to each of these excitation bands for attenuation. In the illustrated example, two excitation bands can thus be completely attenuated, i.e. to the zero value, or even partially attenuated. For each filter draw 21a, 21b a drive signal is produced for the associated
20 motor 25a, 25b. Each filter draw 21a, 21b is continuously shifted via the associated motor 25a, 25b so that a filter 23 selectively acting on the assigned excitation band is inserted in the illumination beam path. Then the filter draw 21a or 21b is shifted further until a surface region of the inserted filter 23 has a transmission factor in the illumination beam path through which the assigned excitation band is attenuated to the intensity setpoint
25 value. In this way, all excitation bands are set to their intensity setpoint values.

Different embodiments of filter draw assembly 20 or the filter draw 21 and the associated spectral transmission curves of the filters arranged on them are explained below.

A filter draw 21 for a multiband fluorescence microscope having two excitation bands A and D is depicted in Figure 2a. Applied to a rectangular glass plate 27 as different vapor deposition layers are a long-pass filter 29 for the intensity reduction of the short-wave excitation band A and a short-pass filter 28 for the intensity reduction of the long-wave excitation band D. They are located on the ends of the glass plate 27, and between them is the blank aperture 22 in the center of filter draw 21. Its cross-section is equal to the beam diameter x in the aperture diaphragm 5 of the illumination beam path. Filter draw 21 can be continuously shifted by positioning means (not shown) longitudinally in both directions parallel to the aperture diaphragm plane 24. The movement is indicated by a double arrow.

Short-pass filter 28 and long-pass filter 29 are each applied in this version as a connected vapor-deposition layer. The transmission of the two filters 28, 29 is largest right next to blank aperture 22 and gets smaller as the distance from blank aperture 22 increases. To this end next to blank aperture 22 filter draw 21 is not completely covered with filters 28, 29, but rather the portion of the filter area increases as one moves from blank aperture 22 toward the end of filter draw 21.

Thus, the vapor-deposition layers on the ends of filter draw 21 form a rectangle with a minimum edge length equal to the beam diameter x . If this vapor-deposited rectangular area completely covers the illumination beam path, the smallest percentile is thus set and the accompanying excitation band is completely attenuated, i.e., to the zero value. The

vapor-deposited rectangular area borders the base of vapor-deposited triangular area, whose opposing corner faces blank aperture 22.

Via the vapor-deposited triangular areas, which always cover the beam diameter only partially, the transmission factor of short-pass filter 28 and long-pass filter 29 in each case diminishes as one moves from blank aperture 22 toward the ends of filter draw 21. By inserting any fractional area of short-pass filter 28 or of long-pass filter 29, any desired transmission factor of the filter 28, 29 in the illumination beam path can be realized and thus one or the other excitation band can be weakened or faded out according to preference.

A filter draw 21 for a multiband fluorescence microscope according to the invention having two excitation bands A and D is likewise depicted in Figure 2b. Applied to a rectangular glass plate 27 are a short-pass filter 28 for filtering the long-wave excitation band A and a long-pass filter 29 for filtering the short-wave excitation band D as different vapor-deposition layers.

Here, as in Figure 2a, the vapor-deposition layers in the direction of blank aperture 22 have a triangular contour. However, the triangles in this case are each not vapor-deposited as connected areas, but rather as small area elements 30 of equal size. In this way a longer adjustment range with higher transmissions is achieved for the two filters 28, 29. At the ends of filter draw 21, the vapor-deposition layers form a rectangle having a minimum edge length equal to the beam diameter x and thus a region of the least transmission with which the assigned excitation band can be attenuated to zero.

Another filter draw 21 for a multiband fluorescence microscope according to the invention having two excitation bands A and D is depicted in Figure 2c. Applied to a rectangular glass plate 27 are a short-pass filter 28 for filtering the long-wave excitation band A and a long-pass filter 29 for filtering the short-wave excitation band D as different
 5 vapor-deposition layers. Located between them is a blank aperture 22.

The filters 28, 29 are vapor-deposited in this version of filter draw 21 next to blank aperture 22 as separate area elements 30 formed in any way desired (here circular).

10 In this context, the size of area elements 30 and thus the portion of the area of filter draw 21 increases when moving from blank aperture 22 to the ends of filter draw 21. The transmission factor of filters 28, 29 is largest next to blank aperture 22 and diminishes when moving in the direction toward the ends of filter draw 21. On both ends of filter draw 21, each of the two filters 28, 29 have a completely vapor-deposited area with the
 15 smallest transmission factor, said area having at least the diameter x of the beam path.

Another filter draw 21 for a multiband fluorescence microscope according to the invention having two excitation bands A and D is depicted in Figure 2d. Applied to a rectangular glass plate 27 are a short-pass filter 28 for filtering the long-wave excitation
 20 band A and a long-pass filter 29 for filtering the short-wave excitation band D as different vapor-deposition layers. Located between them is a blank aperture 22.

In this embodiment the vapor-deposition layers are applied as strip-shaped area elements 30, the width of the strips increasing as one moves from the center to the ends of filter
 25 draw 21. In this way the transmission diminishes as one moves from the blank aperture 22 toward the ends of filter draw 21. Also here the two filters 28, 29 each have a

completely vapor-deposited surface with the lowest possible transmission factor at both ends of filter draw 21, said surface having at least the diameter x of the beam path.

The spectral transmission curves of the short-wave excitation band A and the long-wave excitation band D, as well as the transmission curves T_A , T_D of the associated selective filter, thus the long-pass filter for the short-wave excitation band A and the short-pass filter for the long-wave excitation band D, are depicted in Figure 2e as a function of the light wavelength λ . Filters A and B are selected so that they only selectively filter out the associated excitation band for each but allow the remaining wavelength ranges to pass through unhindered.

Figure 3a shows a rectangular filter draw 21 of a two-piece filter draw assembly 20 for a multiband fluorescence microscope having three excitation bands A, B, D. In this embodiment filter draw assembly 20 consists of two rectangular filter draws 21 identical to said bands that are tightly inserted in the illumination beam path one behind the other next to the aperture diaphragm plane 24.

Provided in the center of filter draw 21 is the blank aperture 22 having diameter x of illumination beam path 2. Applied as vapor-deposition layers on the two ends of filter draw 21 are two different combinations of two or three selective filters 28, 29, 31 for excitation bands A, B, D. The two filters 28, 31 or 29, 31 on one end are in each case arranged next to one another so that both filters border each other and blank aperture 22. In this context each filter 28, 29, 31 has an area of least transmission having at least the diameter x of illumination beam path 2. As a result filter draw 21 has at least a width of $2x$ on its short end.

The transmission factor of filters 28, 29, 31 diminishes as one moves in the longitudinal direction of filter draw 21, thus in the direction of shift from blank aperture 22 toward the ends. This is realized in the example represented here in that the vapor-deposited surface of each filter 28, 29, 31 increases as one moves from blank aperture 22 toward the end of
 5 filter draw 21.

Separate positioning means 25 for shifting filter draw 21 parallel to aperture diaphragm plane 24 in both the longitudinal and transverse directions are provided for each of the two identical shifters 21 of the filter draw assembly. As a result it is possible to insert
 10 filters 28, 29, 31, individually or in combination, completely or with only a portion of their surface in the illumination beam path.

Figure 3b shows a circular filter draw 21 of a two-piece filter draw assembly 20 for a multiband fluorescence microscope according to the invention having three excitation
 15 bands A, B, D. In this advantageous embodiment of the multiband fluorescence microscope according to the invention, filter draw assembly 20 consists of two circular filter draws 21 identical to said bands that are tightly inserted in the illumination beam path one behind the other next to the aperture diaphragm plane 24. Provided in the center of each filter draw 21 is blank aperture 22. Three selective filters 28, 29, 31 for excitation
 20 bands A, B, D are arranged bordering said opening as vapor-deposited ring sectors that border each other.

Filters 28, 29, 31 each possess a transmission factor that decreases as radius increases and is largest next to blank aperture 22. This is accomplished, for example, by virtue of filters
 25 28, 29, 31 not being fully vapor-deposited. Instead, vapor-deposited area elements 30 that get wider as they extend radially and that have non-vapor-deposited surface regions are

applied in between them next to blank aperture 22 so that the portion of vapor-deposited surface of each filter 28, 29, 31 increases as the radius increases.

Using separate positioning means (not shown here), each of the two identical filter draws

5 21 of filter draw assembly 20 can be shifted individually and parallel to aperture diaphragm plane 24. In this case it can either be laterally shifted in one direction and also rotated (as indicated in Figure 3b) or, alternatively, shifted within a plane in two directions. As a result any desired fractional area of filter draw 21, for example blank aperture 22 or one of the filters 28, 29, 31 having the desired transmission factor, can be
10 inserted in the illumination beam path. By arranging two equal filter draws 21 in the illumination beam path, any two of the three filters 28, 29 31 can be inserted simultaneously in the illumination beam path so that two of the three excitation bands can be simultaneously weakened to the predetermined setpoint values or completely faded out.

15 Figure 3c shows an especially advantageous embodiment of a filter draw 21 for a two-piece filter draw assembly 20 of a multiband fluorescence microscope according to the invention having three excitation bands A, B, D. In this embodiment of the multiband fluorescence microscope, filter draw assembly 20 consists of two circular filter draws 21
20 identical to said bands that are inserted in the illumination beam path tightly in succession next to aperture diaphragm plane 24.

The filters 28, 29, 31 for the excitation bands A, B, D are advantageously arranged on the filter draw 21 such that it only has to be turned but not shifted (rotary movement is
25 indicated). This simplifies the structure of the mechanical or even motorized positioning means, which are separately assigned to each of the two filter draws 21 (not shown).

In order to accomplish this, each filter draw 21 is designed with a circular shape, divided into six sectors and arranged pivoted around its center. The center of the circle lies outside the illumination beam path. Each sector can be turned by rotating filter draw 21 into the illumination beam path and cover it completely. The beam cross section with the diameter x is inscribed.

Each second sector is a blank aperture 22. Applied, for example glued on or vapor-deposited, in between each is one of the three filters 28, 29, 31 for the three excitation bands A, B, D. Each filter 28, 29, 31 exhibits an increase of the transmission factor inside its sector in one of the two directions of rotation, for example via different surface coverage with vapor-deposited area elements. As a result each filter 28, 29, 31 has next to the one neighboring blank aperture 22 a maximum transmission factor and next to the other neighboring blank aperture 22 a minimum transmission factor. By rotating the filter draw 21, one of the blank apertures 22 or one of the filters 28, 29, 31 with the desired transmission or a combination of the two can be inserted in the illumination beam path. The use of two identical filter draws 21 as a filter draw assembly makes it possible to attenuate up to two of the three excitations bands A, B, D simultaneously and independently of each other to the desired setpoint value.

Figure 3d shows the spectral transmission curves of the short-wave excitation band A, of the long-wave excitation band D and an intermediate excitation band B as a function of the light wavelength λ .

Furthermore, the transmission curves T_A , T_B , T_D of the accompanying selective filters are depicted, thus the long-pass filter for the short-wave excitation band A, of the short-pass

filter for the long-wave excitation band D and a selective filter for the excitation band B. The filters are selected so that they only selectively filter out the associated excitation band, but the remaining wavelength ranges pass through unhindered.

- 5 Figure 4a shows a circular filter draw 21 for a three-part filter draw assembly 20 of a multiband fluorescence microscope according to the invention having four excitation bands A, B, C, D. In this embodiment of the multiband fluorescence microscope according to the invention, filter draw assembly 20 consists of three circular filter draws identical to said bands that are tightly inserted in the illumination beam path one behind
10 the other next to the aperture diaphragm plane 24. Each filter draw 21 has a blank aperture 22 in the center. In addition four selective filters 28, 29, 31, 32 for the excitation bands A, B, C, D, are vapor deposited as ring sectors that border each other.

- The transmission factor of the filters 28, 29, 31, 32 diminishes as the radius increases and
15 is largest next to the blank aperture 22. This is achieved, for example, by vapor-depositing the filters 28, 29, 31, 32 not completely, but as ring-shaped area elements 30 with non-vapor-deposited areas with vapor-deposition between them, whereby the portion of the vapor-deposited area of each filter 28, 29, 31, 32 increases as the radius increases.

- 20 Separate positioning means (not shown) are assigned to each of the three filter draws 21 of filter draw assembly 20. Using these positioning means, each filter draw 21 can be moved individually and parallel to aperture diaphragm plane 24. In this case it can be shifted laterally in one direction and also rotated (as indicated in Figure 4a) or alternatively shifted in two directions within one plane.

25

As a result, any fractional area of filter draw 21, thus the blank aperture 22 or any fractional area filters 28, 29, 31, 32, can be inserted in the illumination beam path. By arranging three equal filter draws 21 in the illumination beam path, any three of the four filters 28, 29, 31, 32 can be simultaneously inserted in the illumination beam path. As a
5 result up to three of the four excitation bands A, B, C, D can be weakened to the predetermined setpoint value or even faded out completely.

Figure 4b shows an especially advantageous embodiment of a filter draw 21 for a three-part filter draw assembly 20 for a multiband fluorescence microscope according to the
10 invention having four excitation bands A, B, C, D. In this embodiment of the multiband fluorescence microscope according to the invention, filter draw assembly 20 consists of three circular filter draws 21 identical to said bands and are inserted in the illumination beam path tightly in succession next to aperture diaphragm plane 24.

15 The filters 28, 29, 31, 32 for the excitation bands A, B, C, D are arranged on the filter draw 21 such that it only has to be rotated, but does not have to be moved (rotating movement is indicated). That simplifies the design of the mechanical or even motorized positioning means that are separately assigned to each of the three filter draws 21 (not shown).

20 This is achieved by virtue of each filter draw 21 being designed with a circular shape, divided into eight sectors and mounted pivoted around its center. The center of the circle is outside the illumination beam path. By rotating the filter draw 21, each sector can be brought to totally or partially cover the illumination beam path. The beam cross section
25 with the diameter x is inscribed.

- Every second sector is a blank aperture 22. In between them one of the four filters 28, 29, 31, 32 is applied, e.g., glued or vapor-deposited, for the three [sic] excitation bands A, B, C, D. Each filter 28, 29, 31, 32 exhibits an increase of the transmission factor inside its sector in one of the two directions of rotation, for example, via different area coverage.
- 5 As a result each filter 28, 29, 31, 32 has a maximum transmission factor next to the one neighboring blank aperture 22 and a minimum transmission factor next to the other neighboring blank aperture 22.
- By rotating filter draw 21, one of blank apertures 22 or one of filters 28, 29, 31, 32 having
- 10 the desired transmission of a combination of both can be inserted in the illumination beam path. By using three filter draws 21, up to three of the four excitation bands A, B, C, D can be attenuated to the desired setpoint value simultaneously and independently of one another.
- 15 In Figure 4c, the spectral transmission curves of the short-wave excitation band A, of the long-wave excitation wave excitation band D and of the two excitation bands B and C between them are represented as a function of the light wavelength λ . Furthermore, the transmission curves T_A , T_B , T_C , T_D are specified for the associated selective filter, thus the long-pass filter for the short-wave excitation band A, the short-pass filter for the long-
- 20 wave excitation band D, a selective filter for the excitation band B and a selective filter for the excitation band C. Filters A, B, C, D are selected so that they only selectively filter out the particular associated excitation band, but the remaining wavelength ranges pass through unhindered.

For more than four excitation bands, the filter draw assembly 20 must be expanded accordingly. However, it is becoming increasingly difficult to place the individual filters close enough next to aperture diaphragm 5.

5

10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

Reference symbol list

	1	Light source
	2	Optical axis of the illumination beam path
5	3	Collector
	4	First lens member
	5	Aperture diaphragm
	6	Second lens member
	7	Radiant field stop
10	8	Third lens member
	9	Excitation filter
	10	Beam splitter
	11	Objective
	12	Fluorescence object
15	13	Specimen stage
	14	Output filter
	15	Tube lens
	16	Intermediate image plane
	17	Eyepiece on tube (17')
20	18	Video camera
	19	Image analysis system
	20	Filter draw assembly
	21	Filter draw (also 21a, 21b)
	22	Blank aperture
25	23	Selective filter(s)
	24	Aperture diaphragm plane

- 25 Positioning means (25a, 25b motors)
- 26 Control electronics
- 27 Glass plate
- 28 Short-pass filter for the long-wave excitation band D (λ_4)
- 5 29 Long-pass filter for the short-wave excitation band A (λ_1)
- 30 Area element(s)
- 31 Subtractive filter for excitation B(λ_2)
- 32 Subtractive filter for excitation C(λ_3)
- x Beam diameter in the aperture diaphragm plane 24

10

Claims

1. A method for the individual adaptation of excitation intensities in a multiband fluorescence microscope having several spectrally different excitation bands that are simultaneously converted from an fluorescence object (12) into fluorescence bands having fluorescence intensities,
5 wherein
- a) the fluorescence intensities of the individual fluorescence bands in the microscopic image are determined and compared to intensity setpoint values larger than or equal to zero,
10
- b) for each excitation band that is assigned to a fluorescence intensity deviating from the intensity setpoint values, a selective filter (23; 28, 29, 31, 32) is brought into the illumination beam path.
- c) and the transmission factors of the individual filters (23; 28, 29, 31, 32) in effect in the illumination beam path are continuously adjusted so that by attenuating the associated excitation bands, all fluorescence intensities are adjusted to their intensity setpoint values.
15
2. The method as recited in Claim 1,
20 wherein
- the setpoint values for the different fluorescence intensities are all equal to the lowest fluorescence intensity.

3. The method as recited in Claim 1,
wherein
at least one of the setpoint values for the different fluorescence intensities is equal
to zero.

5

4. The method as recited in Claim 1,
wherein
for some fluorescence intensities the setpoint values are equal to zero and for the
rest are equal to the lowest fluorescence intensities.

10

5. The method as recited in Claim 1,
wherein
the different fluorescence intensities are visually determined.

15

6. The method as recited in Claim 1,
wherein
the different fluorescence intensities are determined using an intensity meter.

20

7. The method as recited in Claim 6,
wherein
the different fluorescent intensities are determined using a CCD or a video camera
(18) having an image analysis system (19).

8. The method as recited in Claim 1,
wherein

- a) modifications of the fluorescence intensities are continuously determined,
- b) and by repeated adaptation of the transmission factors of the filters (23;
28, 29, 31, 32) in effect in the illumination beam path, the fluorescence
intensities are always brought back to their setpoint values.

5

9. The method as recited in Claim 2,
wherein

- a) modifications of the fluorescence intensities are automatically
continuously determined,
- b) and by automatic continuous adaptation of the transmission factors of the
filters (23; 28, 29, 31, 32) in effect in the illumination beam path, the
fluorescence intensities are always kept at their setpoint values.

10

15

10. A multiband fluorescence microscope for utilizing the method as recited in Claim 1, including an illumination beam path having a light source (1), a collector (3), a multiplicity of lens members (4, 6, 8), an aperture diaphragm (5), a radiant field diaphragm (7), an excitation filter (9) for simultaneous production of several excitation bands of different light wavelengths and a filter element to affect these excitation bands, also including a beam splitter (10) and an objective (11) that directs the illumination beam path onto a fluorescence object (12) on a specimen stage (13) and projects it through the beam splitter (10), an output filter (14) and a tube lens (15) into an intermediate image plane (16),
- wherein
- a) a filter draw assembly (20) made of individually movable, tightly spaced filter draws (21; 21a, 21b) is inserted perpendicular in the illumination beam path tightly next to the aperture diaphragm (5),
 - b) a selective filter (23; 28, 29, 31, 32) is provided on each filter draw (21) for each excitation band that has surface regions with high and low transmission factors,
 - c) the surface region having the lowest transmission factor has a minimum diameter equal to the beam diameter (x) to completely cancel the excitation band,
 - d) a blank aperture (22) having the beam diameter (x) is arranged next to each filter (23; 28, 29, 31, 32),
 - e) the transmission factor of each filter (23; 28, 29, 31, 32) diminishes as one moves further away from the blank aperture (22),
 - f) and separate positioning means (25), with which any surface region of the filter draw (21) can be inserted in the illumination beam path, are assigned to each filter draw (21).

11. The fluorescence microscope as recited in Claim 10,
wherein
to affect a number n of excitation bands
- 5 a) the filter draw assembly (20) consists of $n-1$ filter draws (21) on separate,
tightly spaced $n-1$ layers/planes parallel to the aperture diaphragm plane
(24),
- b) each filter draw (21) has at least one blank aperture (22) and n selective
filters (23; 28, 29, 31, 32) for the n excitation bands.
- 10 12. The fluorescence microscope as recited in Claim 10,
wherein
to affect two excitation bands A, D a single filter draw (21) is provided,
- 15 a) that has on its one end a long-pass filter (29) to weaken the intensity of the
short-wave excitation band A,
- b) that has on its other end a short-pass filter (28) to weaken the intensity of
the long-wave excitation band D,
- c) and has the blank aperture (22) in between them,
- 20 d) and positioning means (25) for continually shifting the filter draw (21) are
mounted parallel to the aperture diaphragm plane (24).

13. The fluorescence microscope as recited in Claim 11,
wherein

- 5 a) the filter draw (21) has a transparent, rectangular glass plate (27) on whose
ends the short-pass filter (28) and the long-pass filter (29) applied as vapor
deposition layers are opposite one another,
- b) and the short-pass filter (28) and the long-pass filter (29) each have an
increasing portion of the vapor-deposited glass surface, and as a result a
decreasing transmission as one moves from the blank aperture (22) toward
the ends of the filter draw (21).

10

14. The fluorescence microscope as recited in Claim 12,
wherein

the areas of the two vapor-deposition layers at the ends of the filter draw (21) each
have the form of a rectangle having a minimum edge length equal to the beam
15 diameter (x) against which the base of a vapor-deposited, isosceles triangle area
borders in the direction of the blank aperture (22).

15

15. The fluorescence microscope as recited in Claim 12,
wherein

20 the vapor-deposition layers are applied neither wholly or in part as connected are,
but rather as area elements (30) whose size or distances are selected differently as
one moves in the direction of shifting from the blank aperture (22) toward the
ends.

16. The fluorescence microscope as recited in Claim 10,
 wherein
 a two-piece filter draw assembly (20) is provided to affect three excitation bands
 A, B, D,
 - 5 a) each filter draw (21) having a circular blank aperture (22) in the center,
 - b) three selective filters (28, 29, 31) for the excitation bands A, B, D having a
 transmission factor that diminishes as the radius increases are arranged as
 sectors around the center,
 - 10 c) and separate positioning means (25), with which the filter draws (21) can
 be shifted independently of each other parallel to the aperture diaphragm
 plane (24) and or rotated, are assigned to each filter draw (21).
17. The fluorescence microscope as recited in Claim 16,
 wherein
 15 to achieve a transmission factor that diminishes as the radius increases, the portion
 of the vapor-deposited area of the filter (28, 29, 31) increases as the radius
 increases.

18. The fluorescence microscope as recited in Claim 10,
wherein

a two-piece filter draw assembly (20) made of two circular filter draws (21) is
provided to affect three excitation bands A, B, D,

- 5 a) each filter draw (21) being divided into six sectors covering the
illumination beam path and being arranged around its center situated
outside the beam path,
- 10 b) every second sector being a blank aperture (22) and with one of the three
selective filters (28, 29, 31) for the excitation bands A, B, D being situated
between each pair.
- c) each filter (28, 29, 31) exhibiting an increase of the transmission factor in
one of the two directions of rotation.
- 15 d) and separate positioning means (25) being provided for rotating each filter
draw (21) around its center and parallel to the aperture diaphragm plane
(24).

19. The fluorescence microscope as recited in Claim 10,
wherein

a two-piece filter draw assembly (20) made of two rectangular filter draws (21) is
provided to affect three excitation bands A, B, D,

- 5 a) each filter draw (21) having a blank aperture (22) in the center and having
at both of its ends two different combinations of two out of three of the
selective filters (28, 29, 31) for the excitation bands,
- 10 b) the transmission factor of the filters (28, 29, 31) diminishing in the
longitudinal direction of the filter draw (21) moving from the blank
aperture (22) toward the ends,
- c) and separate positioning means (25) being arranged for shifting the filter
draw (21) in the longitudinal direction and parallel to the aperture
diaphragm plane (24).

15 20. The fluorescence microscope as recited in Claim 10,
wherein

a three-piece filter draw assembly (20) made of three circular filter draws (21) is
provided to affect four excitation bands A, B, C, D,

- 20 a) each filter draw (21) having a blank aperture (22) in the center and four
selective filters (28, 29, 31, 32) situated around said center for the
excitation bands as vapor-deposited ring sectors that border each other and
have a transmission factor that decreases as the radius increases,
- b) and separate positioning means (25) being provided parallel to the aperture
diaphragm plane for shifting and/or rotating each filter draw (21).

25

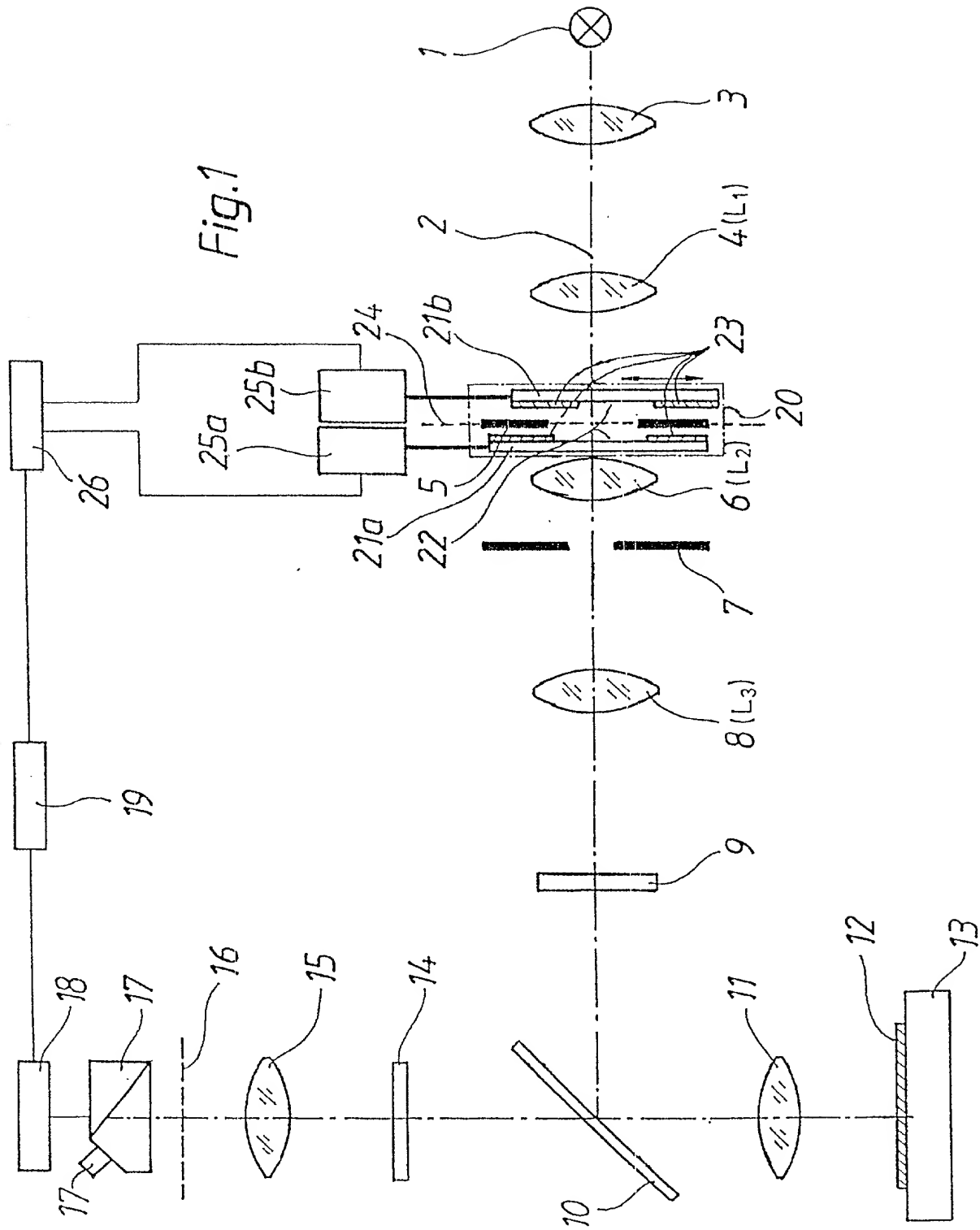
21. The fluorescence microscope as recited in Claim 10,
wherein

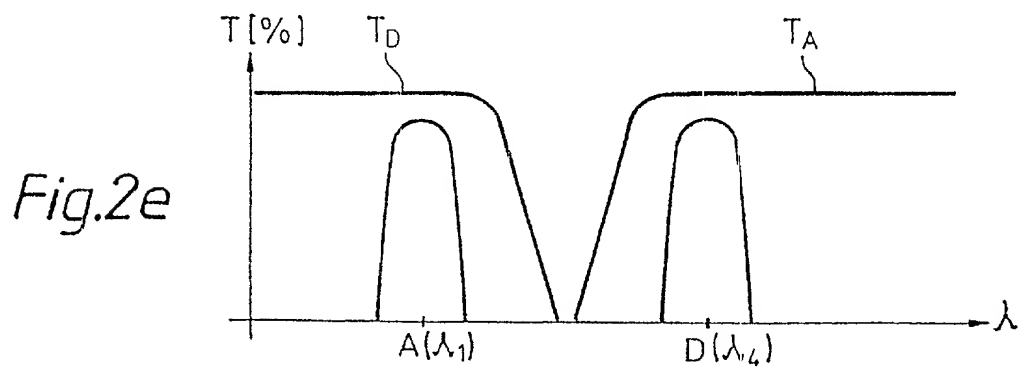
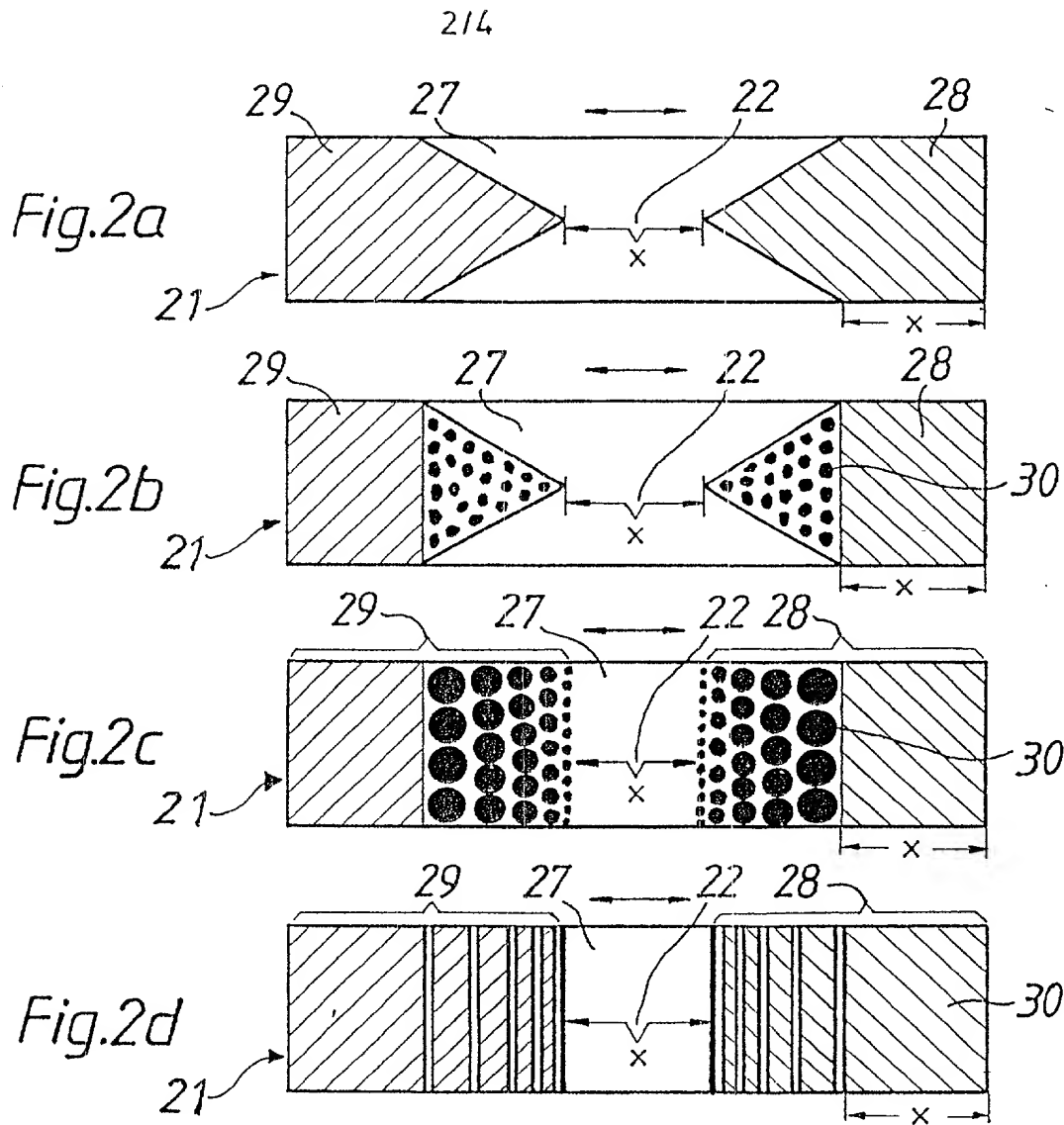
a three-piece filter draw assembly (20) made of three circular filter draws (21) is
provided to affect four excitation bands A, B, C, D,

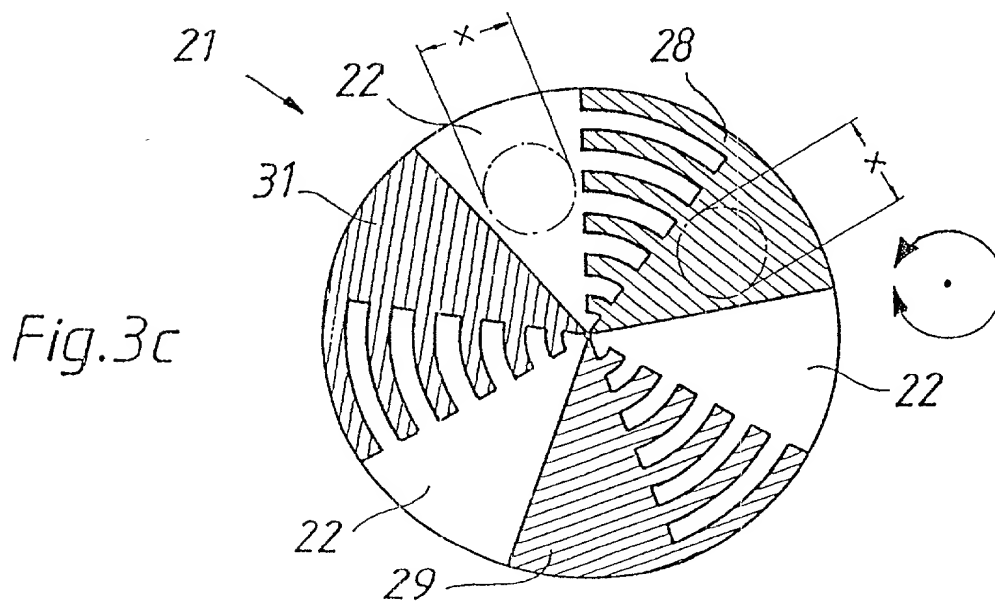
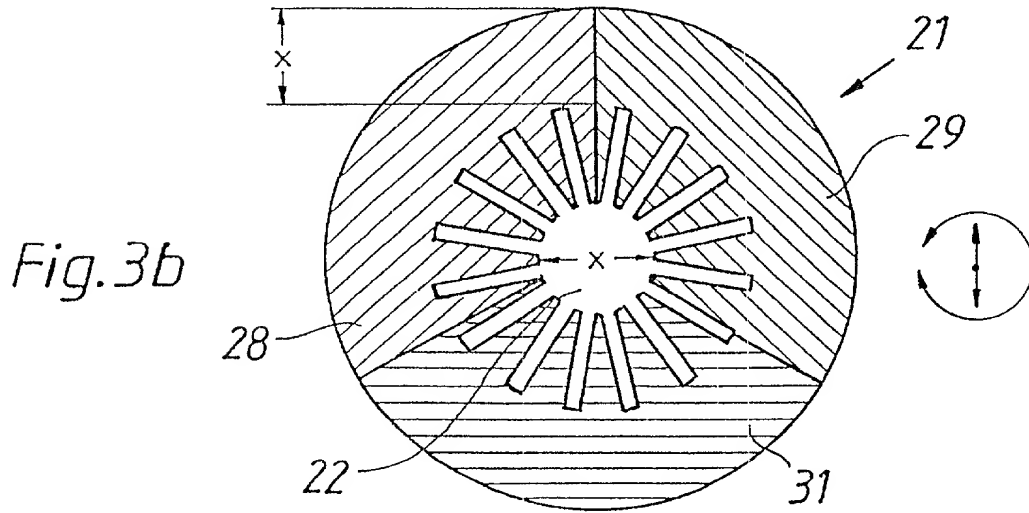
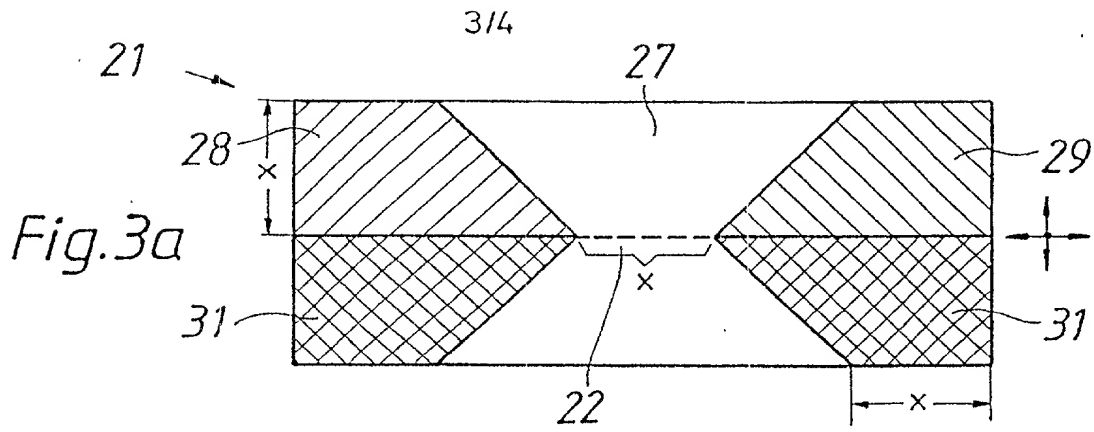
- 5 a) each filter draw (21) being divided into eight sectors covering the
illumination beam path and being located pivoted around its center, which
lies outside the beam path,
- b) every second sector being a blank aperture (22) and in between them being
10 situated one of the four selective filters (28, 29, 31, 32) for the excitation
bands,
- c) each filter (28, 29, 31, 32) showing an increase in the transmission factor
in one of the two directions of rotation,
- d) and separate positioning means (25) being provided for rotating each filter
15 draw (21) around its center and parallel to the aperture diaphragm plane
(24).

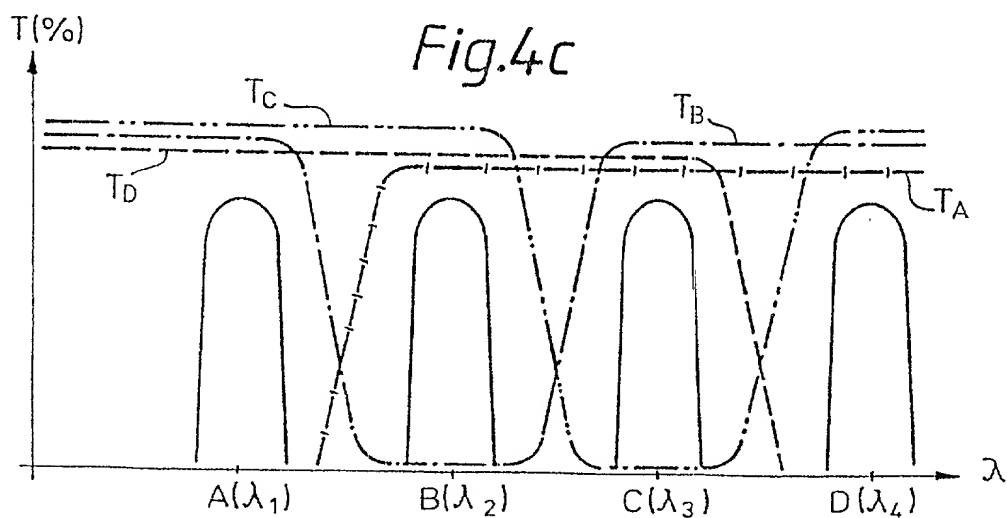
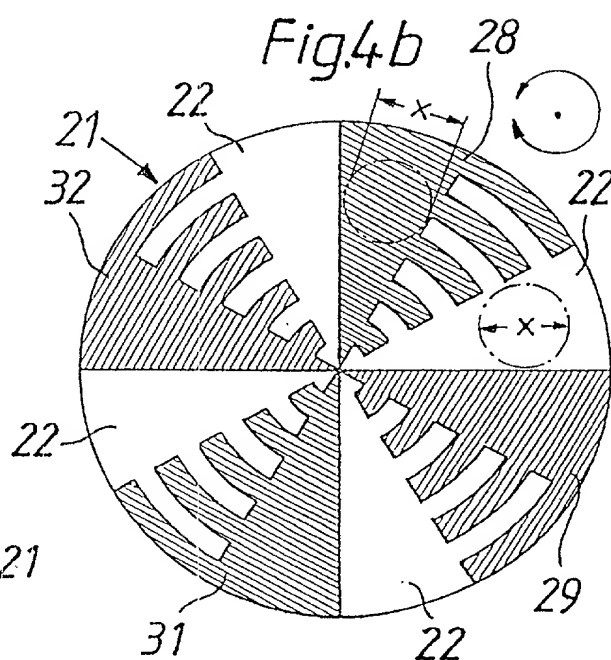
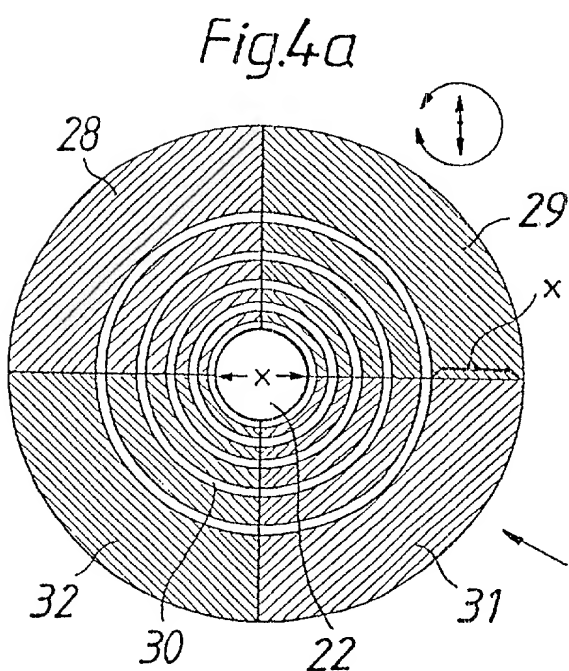
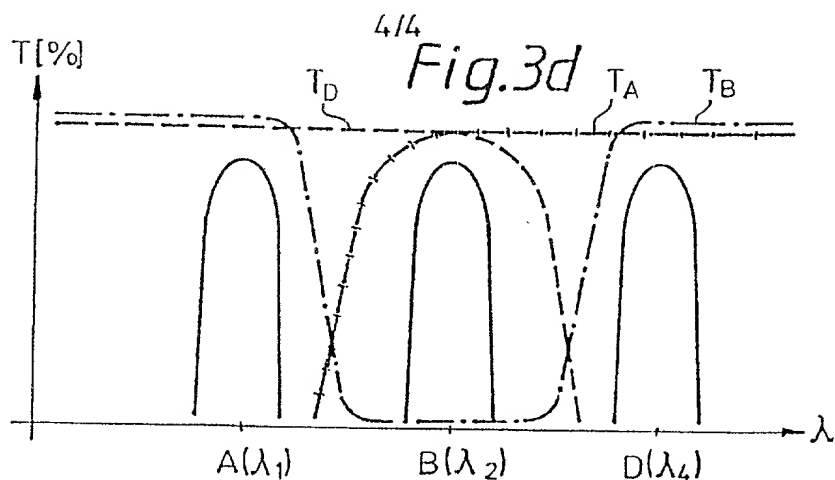
ABSTRACT

The invention relates to a method for the individual adaptation of excitation intensities in a multiband fluorescence microscope with several excitation bands that are different in their spectrums and with associated fluorescence bands. The intensities of the individual
5 fluorescence bands in the microscope image are determined and compared with standard intensity values greater than or equal to zero. For every excitation band that is assigned to a fluorescence intensity deviating from the standard intensity values a selective filter (23; 28, 29, 31, 32) is introduced into the illumination optical path. The transmission degree of the illumination optical path is variably adjusted in such a manner that by
10 attenuating the excitation band the associated fluorescence intensity is adjusted to its standard intensity value. A multiband fluorescence microscope for carrying out the method has a set of filter slides (20) close to the aperture diaphragm (5) which set consists of individually displaceable, closely spaced apart filter slides (21; 21a, 21b) with selective filters (23; 28, 29, 31, 32) with a continuously adjustable transmission degree
15 for every excitation band and with at least one free opening (22). The invention also relates to various advantageous embodiments of the set of filter slides (20).









Declaration and Power of Attorney for Patent Application
Erklärung für Patentanmeldungen mit Vollmacht
German Language Declaration

Als nachstehend benannter Erfinder erkläre ich hiermit an Eides Statt:

daß mein Wohnsitz meine Postanschrift und meine Staatsangehörigkeit den im nachstehenden nach meinem Namen aufgeführten Angaben entsprechen, daß ich nach bestem Wissen der ursprüngliche, erste und alleinige Erfinder (falls nachstehend nur ein Name angegeben ist) oder ein ursprünglicher, erster und Miterfinder (falls nachstehend mehrere Namen aufgeführt sind) des Gegenstandes bin, für den dieser Antrag gestellt wird und für den ein Patent für die Erfindung mit folgendem Titel beantragt wird:

VERFAHREN ZUR INDIVIDUELLEN ANPASSUNG VON ANREGUNGSINTENSITÄTEN BEI EINEM MULTIBAND-FLUORESCENZ-MIKROSKOP UND MULTIBAND-FLUORESCENZ-MIKROSKOP ZUR DURCHFÜHRUNG DES VERFAHRENS

daren Beschreibung hier beigelegt ist, es sei denn (in diesem Falle Zutreffendes bitte ankreuzen), diese Erfindung

- ☒ wurde angemeldet am **27. November 1999** unter der US-Anmeldenummer oder unter der Internationalen Anmeldenummer im Rahmen des Vertrags über die Zusammenarbeit auf dem Gebiet des Patentwesens (PCT) **PCT/DE99/03768** und am _____ abgeändert (falls zutreffend).

Ich bestätige hiermit, daß ich den Inhalt der oben angegebenen Patentanmeldung, einschließlich der Ansprüche, die eventuell durch einen oben erwähnten Zusatzantrag abgeändert wurde, durchgesehen und verstanden habe.

Ich erkenne meine Pflicht zur Offenbarung jeglicher Informationen an, die zur Prüfung der Patentfähigkeit in Einklang mit Titel 37, Code of Federal Regulations, 1.56 von Belang sind.

Ich beanspruche hiermit ausländische Prioritätsvorteile gemäß Title 35, US Code, 119(a)-(d), bzw. 365(b) aller unten aufgeführten Auslandsanmeldungen für Patente oder Erfinderurkunden, oder 365(a) aller PCT internationalen Anmeldungen, welche wenigstens ein Land ausser den Vereinigten Staaten von Amerika benennen, und habe nachstehend durch ankreuzen sämtliche Auslandsanmeldungen für Patente bzw. Erfinderurkunden oder PCT internationale Anmeldungen angegeben, deren Anmeldetag dem der Anmeldung, für welche Priorität beansprucht wird, vorangeht.

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated next to my name. I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

METHOD FOR THE INDIVIDUAL ADAPTATION OF EXCITATION INTENSITIES IN A MULTIBAND FLUORESCENCE MICROSCOPE AND MULTIBAND FLUORESCENCE MICROSCOPE FOR CARRYING OUT SAID METHOD

the specification of which is attached hereto unless the following box is checked:

- ☒ was filed on **November 27, 1999** as United States Application Number or PCT International Application Number **PCT/DE99/03768** and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International Application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Applications
(Frühere ausländische Anmeldungen)

198 58 206.4 Germany 17/December/1998

App. No. Country Day/Month/Year

App. No. Country Day/Month/Year

Ich beanspruche hiermit Prioritätsvorteile unter Title 35, US Code, 119(e) aller US-Hilfsanmeldungen wie unten aufgezählt.

App. No. Filed:

App. No. Filed:

Ich beanspruche hiermit die mir unter Title 35, US Code, 120 zustehenden Vorteile aller unten aufgeführten US-Patentanmeldungen bzw. 365(c) aller PCT internationalen Anmeldungen, welche die Vereinigten Staaten von Amerika benennen, und erkenne, insofern der Gegenstand eines jeden früheren Anspruchs dieser Patentanmeldung nicht in einer US-Patentanmeldung, bzw. PCT internationalen Anmeldung in in einer gemäß dem ersten Absatz von Title 35, US-Code, 112 vorgeschriebenen Art und Weise offenbart wurde, meine Pflicht zur Offenbarung jeglicher Informationen an, die zur Prüfung der Patentfähigkeit in Einklang mit Title 37, Code of Federal Regulations, 1.56 von Belang sind und die im Zeitraum zwischen dem Anmeldetag der früheren Patentanmeldung und dem nationalen oder im Rahmen des Vertrags über die Zusammenarbeit auf dem Gebiet des Patentwesens (PCT) gültigen internationalen Anmeldetags bekannt geworden sind.

App. No. Filed:

App. No. Filed:

Ich erkläre hiermit, daß alle in der vorliegenden Erklärung von mir gemachten Angaben nach bestem Wissen und Gewissen der Wahrheit entsprechen, und ferner daß ich diese eidesstattliche Erklärung in Kenntnis dessen ablege, daß wissentlich und vorsätzlich falsche Angaben oder dergleichen gemäß Section 1001, Title 18 des US-Code strafbar sind und mit Geldstrafe und/oder Gefängnis bestraft werden können und daß derartige wissentlich und vorsätzlich falsche Angaben die Rechtswirksamkeit der vorliegenden Patentanmeldung oder eines aufgrund deren erteilten Patentes gefährden können.

VERTRETUNGSVOLMACHT: Als benannter Erfinder beauftrage ich hiermit den (die) nachstehend aufgeführten Patentanwalt (Patentanwälte) und/oder Vertreter mit der Verfolgung der vorliegenden Patentanmeldung sowie mit der Abwicklung aller damit verbundenen Angelegenheiten vor dem US-Patent- und Markenamt: (Name(n) und Registrationsnummer(n) auflisten)

Priority Not Claimed
(Priorität nicht beansprucht)

☐

☐

☐

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

I hereby claim the benefit under Title 35, United States Code, 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

Status: Patented/Pending/Abandoned

Status: Patented/Pending/Abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith: (list name and registration number)

George L. Snyder, Jr.
Robert P. Simpson
R. Craig Kauffman
Howard M. Ellis

Reg. No. 37,729
Reg. No. 33,034
Reg. No. 20,362
Reg. No. 25,856

4

Postanschrift:

Send Correspondence to:

Simpson, Simpson & Snyder, L.L.P.
5555 Main Street
Williamsville, New York 14221

Telefonische Auskünfte:

Direct Telephone Calls to:

George L. Snyder, Jr. (716) 626-1564

Full Name of sole or first inventor

Inventor's signature:
Unterschrift des Erfinders:

Date:
Datum:

Albrecht WEISS

Albrecht Weiss

05/17/2001

Residence:

Schillerstrasse 18

Wohnsitz:

D-35440 Linden

Germany

Citizenship:

Germany

Staatsangehörigkeit:

Post Office Address:

Same as above

Postanschrift: